Characterizing Herpes Simplex Virus Type 1 Replication Kinetics, Latency, and Reactivation in the Lip Scarification Model of Infection and Disease

A Dissertation
Submitted to the Faculty
of
Drexel University College of Medicine
by
Kevin Pollard Egan
in partial fulfillment of the
requirements for the degree
of
Doctor of Philosophy
December 2016

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Dedications

This dissertation is dedicated to all those who have encouraged me to pursue a career in science, William McCaffrey, Afeworki Mascio, Ph.D., and Robert Pignolo, M.D., Ph.D. My family has been an eternal source of encouragement through this whole process. I can never thank you enough for the years of support, William R. Pollard, Ph.D., Kathleen L. Egan., Daniel P. Egan, MS and Martha L Egan.

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Characterizing Herpes Simplex Virus type 1 Replication Kinetics, Latency, and Reactivation in the Lip Scarification Model of Infection and Disease

Kevin Pollard Egan

Herpes simplex virus type 1 (HSV-1) is a common human pathogen which infects the majority of adults. The virus establishes a lifelong latent infection in sensory neurons and periodically reactivates to cause recurrent disease. Most new human infections above the neck occur in the lower lip but most animal models of HSV-1 infections do not inoculate through the lip. Here we describe the acute and latent infection events of HSV-1 infections in a lip model of HSV-1 infection and disease. Mice exhibited clinical lesions and pathology consistent with human HSV-1 infections. Mice were able to clear the primary infection, establish latency, and the resultant latently infected tissue was capable of supporting viral reactivation. This HSV-1 infection system was subsequently used to perform initial testing of a novel CRISPR/Cas9 gene editing therapeutic. Overall, these studies have established the basic events of acute and latent infection as well as viral reactivation in mice infected via a lip inoculation.

In addition, these studies have shown the utility of the lip scarification and HSV-1 infection and disease model to study molecular pathogenic events associated with the establishment, maintenance, and reactivation of latent infection and associated disease processes. This model can be used for testing a variety of therapeutics for efficacy with respect to reducing viral replication and pathology in the lip and to minimize or eliminate acute HSV-1 infection and/or remove latent viral genomes in order to develop a cure for viral reactivation and recurrent HSV-1 infections.

CHAPTER 1

Immunological Control of Herpes Simplex Virus Infections

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1.1. ABSTRACT

Herpes simplex virus type 1 (HSV-1) is capable of causing a latent infection in sensory neurons that lasts for the lifetime of the host. The primary infection is resolved following the induction of the innate immune response that controls replication of the virus until the adaptive immune response can clear the active infection. HSV-1-specific $CD8^+$ T cells survey the ganglionic regions containing latently infected neurons and participate in preventing reactivation of HSV from latency. The long-term residence and migration dynamics of the T cells in the trigeminal ganglia appear to distinguish them from the traditional memory T-cell subsets. Recently described tissue resident memory (T_{RM}) T cells establish residence and survive for long periods in peripheral tissue compartments following antigen exposure. This chapter focuses on the immune system response to HSV-1 infection. Particular emphasis is placed on the evidence pointing to the HSV-1-specific $CD8^+$ T cells in the trigeminal ganglia belonging to the T_{RM} class of memory T cells and the role of T_{RM} cells in virus infection, pathogenesis, latency, and disease.

1.2. Introduction to virus and infection cycle

Herpes simplex virus (HSV) type 1 (HSV-1) is a ubiquitous human pathogen capable of causing an infection that lasts for the lifetime of the host as a primary, latent, recurrent, or persistent infection. The HSV-1 genome is a 152-kbp double-stranded linear DNA genome that encodes for upwards of 84 genes (Roizman et al. 2007). The genome is contained within the nucleocapsid, which is surrounded by a heterogeneous group of proteins collectively designated as the tegument. The tegument and nucleocapsid are surrounded by a lipid envelope studded with glycoproteins that are used to bind to and enter new susceptible cells. HSV-1 is a widespread pathogen that can be found in up to 53% of the adult population in the United States, with carriage found to be even higher in selected groups of individuals (Xu et al. 2006). A unique feature of HSV-1 and other human α-herpesviruses (HSV type 2 [HSV-2] and varicella zoster virus; VZV) is the ability to cause a latent infection of sensory neurons innervating peripheral tissues. The latent infection is maintained for the lifetime of the host and can be periodically interrupted by asymptomatic or clinically apparent reactivation events of variable duration and severity. The number of recurrent infections in an individual with HSV is highly variable, whereas reactivation of VZV is seldom observed more than once or twice over the course of a lifetime. The natural history of VZV has been dramatically altered by the availability of vaccines directed at preventing varicella or chickenpox, the primary

disease (Shah et al. 2010), and zoster or shingles, the reactivated form of the disease (Oxman et al. 2005). An effective vaccine directed against with HSV-1 or HSV-2 has yet to be delivered to the human population to prevent either primary or reactivated disease or as a therapeutic modality (Chentoufi et al. 2012).

The HSV-1 infection cycle begins when the virus replicates in epithelial cells following transmission to a new uninfected host. HSV-1 glycoproteins B (gB) and C (gC) are responsible for virion attachment to host cells by interacting with heparan sulfate proteoglycans (HSPGs) (Shukla and Spear, 2001). Filopodia extending out of cells contain high concentrations of HPSGs and viruses have been shown to travel along filopodia to cell bodies in a process termed virus surfing (Oh et al., 2010). Cells lacking HSPGs can still be infected by virions due to the ability of gB to bind to paired immunoglobulin like type 2 receptor alpha (PILRα) (Satoh et al., 2008).Glycoprotein B is highly conserved and is essential for virion attachment and fusion. Glycoprotein C is considered non-essential as viruses lacking gC are still infectious, though they have reduced infection efficiency (Shukla and Spear, 2001).

Following binding to the cell surface, the virus enters through fusion at the plasma membrane or endocytic vesicles. Fusion is mediated through glycoprotein D (gD) interacting with cellular receptors which induces a conformational shift in gD (Carfí et al., 2001). This conformational change causes the formation of the multiprotein fusion

complex which consists of gD, gB, gH, and gL (Campadelli-Fiume et al., n.d.). Glycoprotein D has been shown to be capable of using multiple cellular receptors found on numerous cell types for initiation of fusion. Herpesvirus entry mediator (HVEM) has been shown to be used by gD for entry of HSV-1 into human trabecular meshwork cells (Tiwari et al., 2005). Specific isoforms of 3-O sulfated heparan sulfate were shown to be used for entry into primary corneal fibroblasts (Shukla et al., 1999). Nectin-1 has been shown to be responsible for entry into epithelial cells and importantly neuronal cell populations (Shukla et al., 2012; Simpson et al., 2005). Additionally PILRα has been shown to enable infection of normally resistant cell populations when they are transfected to express PILRα (Satoh et al., 2008). HSV-1 has also shown to be capable of entering cells through fusion following endocytosis. The mechanism of entry for this pathway has not been fully worked out, but it appears that it could be cell type and/or receptor specific (Nicola and Straus, 2004).

Fusion of the HSV-1 viral envelope with the plasma membrane of susceptible cells delivers the tegument proteins and nucleocapsid into the cytosol of the target cell. Tegument proteins serve to regulate cell processes (Strom and Frenkel, 1987), evade the immune system (Sen et al., 2013), and promote transcription of viral genes. Tegument protein VP16 ($V_{MW}65$, or an α-transinducing factor [α-TIF]) associates with host cell factor 1 (HCF1) and octamer-binding transcription factor 1 (OCT 1) to form a transcription factor complex that strongly promotes the transcription

of immediate-early (IE) or α viral genes (Gerster, 1988). The transcription of HSV-1 genes proceeds in an organized temporal pattern, with α genes being expressed first. The IE gene products serve as activators for the transcription of early or β genes, which encode for proteins necessary for replication of viral DNA. Following amplification of the viral genome, the late or γ genes, which comprise the virion structural components, have been shown to be expressed, as previously reviewed (Roizman et al. 2011). Packaged virions are released and spread to nearby uninfected cells, thereby expanding the amount of infectious virus and facilitating the spread of the infection to neighboring epithelial cells. Perhaps more importantly with respect to the natural history of HSV disease, the virus also infects neighboring peripheral sensory neurons during primary infection setting the stage for the establishment of latent infection.

HSV-1 virions bind to and enter axons and travel in a retrograde direction towards the neuronal cell body, as previously reviewed (Smith 2012). After reaching the nucleus of the neuron, the viral genome is translocated from the capsid docked at the nuclear membrane into the nucleus, where it is thought to circularize in preparation for DNA synthesis. The circular form of the HSV genome has also been shown to be the predominant form of the genome that is maintained during the course of viral latency (Mellerick and Fraser 1987; Su et al. 2002). At the earliest stages of nuclear invasion, there are a number of different viral replication modes that can be followed which have different consequences for the

infected neuron. In some cases, genomic activation of IE genes by the tegument transactivator protein VP16, HCF/OCT, and possibly other factors leads to productive infection with synthesis of infectious virions that can cause the death of at least some neurons with the potential for replication and disease in the periphery after release of the progeny virus from neuronal axons in the periphery. However, in some neurons, productive infection may be prevented or aborted by the presence of neuronal nuclear factors that prevent the activation of the promoters and therefore prevent or abort productive replication and send the virus into a latent state (Bloom et al. 2010; Cliffe et al. 2009; Knipe and Cliffe 2008). It has been proposed that some neurons are more susceptible to productive infection whereas some neuronal types might be either reversibly or irreversibly susceptible to viral latency based on the differential presence of regulatory RNAs or regulatory proteins (Bertke et al. 2012; Bertke et al. 2011; Yang et al. 2000). The latent infection has been characterized as maintenance of the viral genome within the neuron in the absence of production of new cell-free infectious virus. Viral gene expression has been shown to be limited to the latency-associated transcript (LAT) and possibly the low-level expression of other genes (Feldman et al. 2002; Kramer and Coen 1995; Ramachandran et al. 2010). The protein and/or RNA regulatory factors that guide the virus into latency or that maintain the control of the latent state or initiate reactivation are largely unknown but are beginning to come to light (Camarena et al. 2010; Kim et al. 2012;

Thompson and Sawtell 2011). It may also be possible that the greater distance the virus must travel to reach the neuronal nucleus, the lower the level of tegument proteins that will be available for promotion of IE gene expression (Sears et al. 1991) once the capsid has reached the nucleus of the infected neuron. An additional mechanistic theory centers on neuronal sequestration of HCF1 in the cytoplasm instead of the nucleus, in which case this factor would not be available to interact with VP16 in the nucleus to enhance transactivation of early promoters and facilitate productive replication (Kristie et al. 1999). In general, a number of studies have suggested that it is likely that a combination of viral and host cell factors may be responsible for the establishment and maintenance of latent infection of neurons. The latent phase has been theorized to be of evolutionary benefit to the virus as it maintains a stable reservoir of viral genomic information from which periodic reactivations emanate and allows for transmission of infectious virus to new hosts over the lifetime of the infected host.

Following selected stimuli, viral gene expression has been shown to be enhanced, with infectious virus production first in the ganglia and later at peripheral sites. At least a fraction of the newly made capsids in the neuronal nucleus are thought to travel anterograde to the axonal terminal with release into the periphery (Smith et al., 2001). Reactivation of the virus can often be asymptomatic, but replication in peripheral epithelial cells can also lead to symptomatic disease. Herpes labialis has

been shown to be caused by the formation of vesicular lesions resulting from replication in epithelial cells. Rupture of these lesions causes spread of the virus to different anatomical regions or transmission to new hosts. Usually the disease has been shown to be self-limiting in immunocompetent individuals; however, serious disease can occur in immunocompromised individuals (Rowley et al., 1990). Replication in corneal epithelial cells results in corneal scarring and can lead to herpes stromal keratitis, a leading cause of blindness in the United States (Kaye and Choudhary 2006). HSV-1 infection of the brain is rare but can lead to fatal herpes encephalitis in the absence of early therapeutic intervention (Brady and Bernstein 2004).

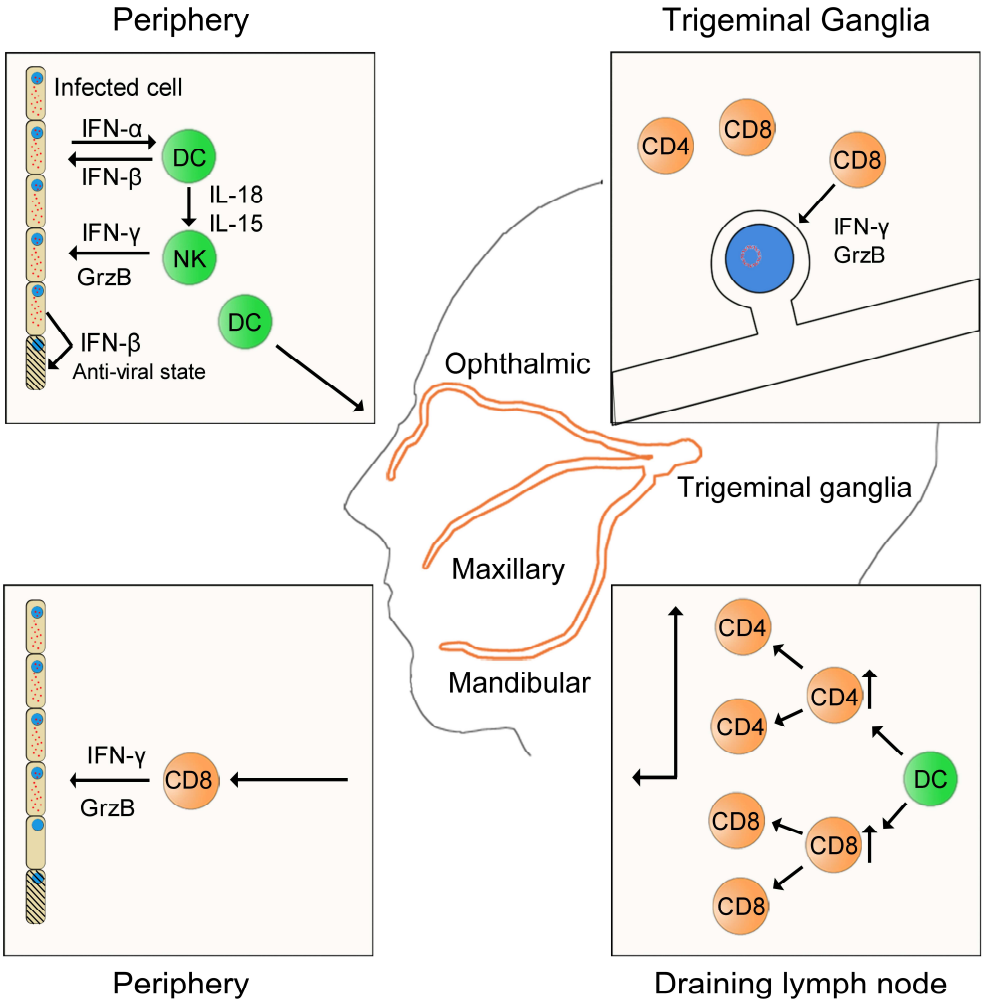
1.3. Role of the immune system in HSV-1 infection

The immune system plays an important role in limiting HSV-1 replication during primary infection and in maintaining the viral genome in a latent state for prolonged periods in the absence of infectious virus production and clinical symptoms. Replication in peripheral epithelial cells causes production and release of type 1 interferons that activate cells of the innate immune system. Activated macrophages secrete interferons and cytokines, which recruit and activate additional cells of the innate immune response to the site of infection. Type 1 interferons induce an antiviral state in uninfected epithelial cells that makes it harder for the virus to replicate in these cells as well. Recruited neutrophils induce apoptosis in infected cells and phagocytose dying cells. Activated natural killer (NK)

cells release interferon-γ and granzymes A and B, which induce apoptosis in infected cells. Dendritic cells (DCs) secrete proinflammatory cytokines, take up antigen, and present viral peptides to cells of the adaptive immune system. The innate immune response limits viral replication in the periphery and presents antigen to the naïve lymphocytes to activate the adaptive immune response. Previous studies have suggested that the principal role of B cells in the immune response to HSV-1 infection is not to produce neutralizing antibodies but instead to present antigen and secrete cytokines (Deshpande et al. 2000). Activated $CD4^+$ and $CD8^+$ T cells play a pivotal role in clearing the primary infection. Additionally, $CD8^+$ T cells are important for maintaining the virus in the latent state, as will be discussed below (Figure 1.1).

Figure 1.1. HSV-1 immune responses. Productive HSV-1 infection occurs in epithelial cells in the oral mucosa. Released virons infect neighboring cells and innervating sensory neurons. The virus travels retrograde to establish latent infection in the trigeminal ganglia. HSV-1 infection is detected in epithelial cells and induces production of interferon-β (IFN-β). IFN-β activates innate immune cells, which in turn secrete IFN-α. Type 1 IFNs induce the antiviral state in an autocrine fashion in the producing cell and in surrounding epithelial cells. Interleukin-18 (IL-18) released from dendritic cells (DCs) activates natural killer (NK) cells that secrete IFN-γ and granzymes A and B (GrzAB). DCs engulf HSV-1 virions and travel to the draining lymph node where they activate naïve B and T cells. Activated T cells travel back to the site of primary infection and the trigeminal ganglia,where they surround infected neurons and prevent replication via secretion of IFN-γ and granzymes A and B.

Figure 1.1.


Periphery
Trigeminal Ganglia
Infected cell
IFN-α
IFN-β
DC
IL-18
IL-15
IFN-γ
GrzB
NK
DC
IFN-β
Anti-viral state
CD4
CD8
CD8
IFN-γ
GrzB
Ophthalmic
Trigeminal ganglia
Maxillary
Mandibular
IFN-γ
GrzB
CD8
CD4
CD4
CD4
DC
CD8
CD8
CD8
Periphery
Draining lymph node

Detection of HSV-1 infection occurs initially within the infected peripheral epithelial cells. Viral pathogen-associated molecular patterns (PAMPs) are detected by pattern recognition receptors (PRRs) within infected cells and induce intracellular signaling, which results in cytokine release and interferon production. Recently, the detection of HSV-1 PAMPs by cellular PRRs has been reviewed (Melchjorsen 2012; Paludan et al. 2011). Extracellular HSV-1 has been detected as a result of gD binding to PRRs on the surface of the host cell prior to membrane fusion (Ankel et al., 1998; Kim et al., 2012). Intracellular PAMPs such as viral nucleic acids are detected either in the cytoplasm or in endosomal compartments.

Viral nucleic acids are the primary PAMPs detected by HSV-infected cells and readily induce expression of proinflammatory cytokines and interferons. The HSV-1 genome has been shown to be composed of double-stranded DNA (dsDNA) that contains unmethylated CpG motifs (Lundberg et al. 2003). CPG motifs have been detected based on the Toll-like receptor 9 (TLR-9) being embedded in the membrane of endosomal compartments. How HSV-1 DNA has been detected within endosomal compartments is not definitively known, but the virus has been shown to be capable of infecting cells through endocytosis in addition to membrane fusion (Rahn et al. 2011). HSV-1 DNA from infected cells can also be endocytosed, where it can be detected by TLR-9 in plasmacytoid DCs (pDCs) and B cells (Bosnjak et al. 2012; Pollara et al. 2003). Cytoplasmic

DNA sensors detect HSV-1 DNA not found in endosomal compartments. DNA-dependent activator of interferon regulatory factors (DAI), interferon-γ-inducible protein 16 (IFI16), DXH9, and DXH36 detect DNA motifs in the cytoplasm. IFI16 has recently been shown to be able to activate and maintain normal levels of interferon in the absence of TLR signaling in corneal epithelial cells (Conrady et al. 2012). DXH9 and DXH36 detect DNA-containing CpG motifs and activate NF-κB and interferon regulatory factor 7 (IRF7), respectively.

Although the HSV-1 virion contains a DNA genome, replication intermediates have been detected by PRRs specific for double-stranded RNA (dsRNA) (Weber et al., 2006). Endosomal dsRNA has been detected by TLR-3, which has been shown to be expressed in many cell types and expression can be induced by type 1 interferons (Tissari et al., 2005). The importance of TLR-3 activation in HSV-1 infection has been illustrated by children with genetically deficient TLR-3, who have greater susceptibility to herpes encephalitis (Guo et al. 2011; Herman et al. 2012; Zhang et al. 2007). Recently Lafaille et al. (2012) demonstrated that TLR-3-deficient CNS cells have reduced intrinsic innate immune activation. Taking dermal fibroblasts from TLR-3-deficient patients and generating induced pluripotent stem cells of neuronal lineage, they demonstrated that TLR-3-negative neurons and oligodendrocytes induce fewer type 1 interferons in response to HSV-1 infection. Cytosolic viral RNA intermediates are detected through retinoic acid inducible gene (RIG)-like

receptors (RLRs) that contain DExD/H box helicase domains (Kato et al. 2008). RIG-I and MDA-5 (melanoma differentiation-associated gene 5) recognize short dsRNA (Schlee et al., 2009) and longer dsRNA higher-order structures (Pichlmair et al., 2009), respectively. RLR signaling induces IRF-3 and NF-κB activation, leading to type 1 interferon production and proinflammatory cytokine production.

The interferon-induced antiviral state has been shown to be important for limiting the immediate viral replication in susceptible cells of the periphery. The difference between the severe response observed in BALB/c mice compared with the controlled response in C57BL/6 mice has recently been attributed to the enhanced innate response present in the C57BL/6 mice (Sheridan et al. 2009). Interferon signaling leads to expression of interferon-stimulated genes ribonuclease L (RNAse L), protein kinase RNA-activated (PKR), and 2′-5′ oligoadenylate synthetase. These antiviral enzymes accumulate in cells in response to interferon binding and have been shown to be ready to act upon signs of virus infection. PKR phosphorylates EIF-2α, a necessary protein for protein translation to proceed. Inhibition of protein translation prevents viral replication and causes viral mRNA transcripts to accumulate. Viral mRNA transcripts are actively degraded by RNAse L, which has been shown to be activated in response to dsRNA and 2′-5′ oligoadenylate synthetase. These mediators of translational inhibition are activated in response to

PAMPs present within infected cells and thus do not necessarily prevent translation in uninfected cells.

Primary recruited immune cell mediators include monocytes, neutrophils, DCs, and NK cells. Attracted neutrophils secrete the antiviral molecule tumor necrosis factor-α (TNF-α), which can induce apoptosis in infected epithelial cells through a caspase-8-dependent pathway. Neutrophils also phagocytose necrotic and apoptotic epithelial cells. Monocytes attracted to the area differentiate into tissue macrophages and phagocytose released virions and apoptotic cells. Macrophages are professional antigen-presenting cells (APCs) that release proinflammatory cytokines and present viral peptides to cells of the adaptive immune response. Macrophages in peripheral tissue sites where initial infection occurs (Cheng et al. 2000; Mott et al. 2007) and in the trigeminal ganglia (Kodukula et al. 1999) serve to limit viral replication until the adaptive immune system can be activated. Macrophages secrete proinflammatory cytokines such as TNF-α, interleukin-6 (IL-6), RANTES (“regulated on activation, normal T cell expressed and secreted”), type 1 interferons, and nitric oxide (NO). NO production has been shown to be initiated by the enzyme inducible nitric oxide synthase (iNOS), which is activated by interferon-γ released from γδ-T, NK, $CD4^+$ T, and $CD8^+$ T cells. NO released from macrophages has been shown to significantly reduce HSV-1 levels when treating in vitro macrophage cultures. When iNOS is

inhibited in mouse models, virus replication has been shown to increase in the trigeminal ganglia (Kodukula et al. 1999).

Dendritic cells play an important role in the immune response by activating NK cells at the site of infection and acting as APCs to activate cells of the adaptive immune system. HSV-1 PAMPS activate immature DCs which differentiate and mature. Mature DCs can activate other immature DCs through the release of type 1 interferons (Pollara et al. 2004). Mature DCs travel to the draining lymph node, where viral peptides are presented to naive B and T cells for activation. Viral peptides can also be presented in the context of HSV-1 infection of DCs through a cross-presentation pathway. HSV-1-infected DCs are unable to mature but are still capable of secreting cytokines that activate uninfected DCs; infected DCs are also induced to undergo apoptosis via downregulation of C-FLIP (cellular FLICE-like inhibitory protein) (Kather et al. 2010). Infected apoptotic DCs are taken up by activated DCs which process the antigen and present peptides to cells of the adaptive immune system (Bosnjak et al. 2012). An additional role of DCs involves the secretion of IL-18, which has been shown to be necessary for the activation of NK cells (Kassim et al. 2009; Reading et al. 2007). Mice lacking DCs exhibit increased HSV-1 neurovirulence during the course of infection based primarily on the limited activation of NK, $CD4^+$ T, and $CD8^+$ T cells (Kassim et al. 2006).

Natural killer cells are crucial to controlling HSV-1 virus levels in the periphery by directly inducing cell death in infected epithelial cells (Grubor-

Bauk et al. 2008). Like $CD8^+$ T cells, NK cells contain preformed granules of perforin, granzyme A, and granzyme B. HSV-1 infection reduces major histocompatibility complex (MHC) class 1 expression in epithelial cells by the action of ICP47, a viral immediate-early gene, which blocks loading of TAP (transporter associated with antigen presentation) and presentation of viral peptides through MHC class 1 (York et al. 1994). The reduction in MHC class 1 has been shown to be a signal that NK cells use to target virus-infected cells (Ravetch 2000). Recently it has been shown that NK cells can also be activated by HSV-1 gD binding to TLR-2 on the surface of NK cells (Kim et al. 2012). Mice have been shown to suffer greater mortality in response to HSV-1 infection when NK cells are depleted (Grubor-Bauk et al. 2008; Williams et al. 1998) or NK survival factor IL-15 is inhibited in the context of HSV-2 infection (Ashkar and Rosenthal 2003).

1.4. $CD8^+$ T-cell response

Antiviral $CD8^+$ T cells exert their effector function through secretion of interferon-γ, TNF-α, perforin, and granzymes after engaging antigens in the context of MHC class I molecules of the target cells. $CD8^+$ T cells induce apoptosis in infected cells by releasing preformed granules or through death receptor signaling. The granules contain perforin, granzymes, and granulysin, which work together to enter the target cell and induce apoptosis. Perforin has been shown to polymerize on the target cell membrane forming a pore, allowing entry of granzymes and granulysin (Liu et al. 1995; Voskoboinik et al. 2005). However, in the

absence of perforin, granzyme B can be internalized by endocytosis into the target cell, possibly by interacting with the mannose-6-phosphate receptor (Motyka et al. 2000). Although perforin and granzymes are both internalized and released into the cytoplasm of the target cell, perforin was found to be essential for inducing apoptosis (Froelich et al. 1996). Granulysin, a small cationic protein, has been shown to be present in the lytic granules of NK cells, NKT cells, helper T cells, and cytotoxic T lymphocytes (CTLs) (Latinovic-Golic et al. 2007). This process has been shown to cause target cell lysis when it interacts with negatively charged cell membrane proteins. Granulysin can also induce apoptosis through the release of cytochrome C (Latinovic-Golic et al. 2007).

Granzymes A and B belong to the serine-protease family and can induce apoptosis in caspase -independent and -dependent ways (Waterhouse et al. 2006b). Granzyme A acts in a caspase independent way to induce apoptosis by cleaving single-stranded DNA and by hydrolyzing histone proteins (Fan et al. 2003; Mueller et al. 2003). It can also cleave the IL-1β propeptide into the active IL-1β (Irmler et al. 1995; Nicola et al. 2005). Granzyme B can induce apoptosis in a caspase-dependent manner by activating procaspase 3 directly or by increasing the permeability of mitochondria and cleaving the Bcl-2 interacting domain (Bid) protein (Metkar et al. 2003; Pinkoski et al. 2001).

$CD8^+$ T cells also respond to viral infections through the production and release of interferon-γ. Interferon-γ promotes presentation of viral

peptides, inhibits viral replication, arrests the cell cycle, and promotes the th1 immune response. Interferon-γ induces expression of immunoproteosomal subunits (Belich et al., 1994; Groettrup et al., 1996; Kelly et al., 1991) which can enhance processing of viral peptides for loading into the MHC class 1 pathway (Sijts and Kloetzel, 2011). Interferon-γ also enhances the class 2 antigen presentation pathway, increasing expression of MHC 2 molecules in both professional and non-professional cells (Handunnetthi et al., 2010). Cells exposed to IFN-γ inhibit viral replication through expression of antiviral genes like PKR which inhibits translation within the cell (Meurs et al., 1990). Additional effects of IFN- γ include increased expression of cyclin dependent kinase inhibitors p21 (Xaus et al., 1999) and p27 (Harvat et al., 1997) to arrest cell cycle progression. Interferon-γ also skews the immune response by promoting differentiation of naïve T cells into Th1 cells (Yoshida et al., 1994).

Death receptor signaling is a caspase-8-dependent process of inducing apoptosis. CD95 ligand (CD95L) on the surface of $CD8^+$ T cells binds with the CD95 receptor to initiate signaling. The CD95 receptor contains a death effector domain that is a binding site for procaspase 8. Procaspase 8 has been shown to be cleaved upon binding to the death effector domain, forming the active caspase 8. Activated caspase 8 can then directly cleave procaspase 3, the primary downstream mediator of apoptosis. Both granzyme B and activated caspase 8 can cleave Bid to

the truncated form (tBid). The activated tBID can induce release of cytochrome C from the mitochondria and induce apoptosis through the intrinsic pathway (Korsmeyer et al. 2000; Waterhouse et al. 2006a; Waterhouse et al. 2006b).

The mechanisms described above allow virus-specific CTLs to efficiently eliminate virus-infected cells from the body by inducing apoptosis and thereby limit the virus capacity to produce new virions. This mechanism of clearance has been shown to be an effective response to viruses in peripheral tissues where apoptotic cells can be replaced by mitogenic precursors. However, the neurons that reside in the nervous system are nonmitogenic. This presents a problem for the body wherein clearance of virus-infected neurons cannot be replaced (Okouchi et al. 2007). Indeed, previous results have indicated that granule-mediated apoptosis by $CD8^+$ T cells may be involved in the control the latent HSV-1 infection; however, latently infected neurons are rarely induced into apoptosis.

Virus-specific $CD8^+$ T cells infiltrating into the trigeminal ganglia are capable of preventing reactivation and maintaining HSV-1 in the latent state (Liu et al. 2000). CTLs infiltrate into the trigeminal ganglia, surround infected neurons, form immunological synapses, and release preformed granules that can be detected within target cells. Yet these molecules do not induce apoptosis of latently infected neurons (Knickelbein et al. 2009). Granzyme A was shown to act in a noncytolytic capacity to limit the

spread of HSV-1 to other neurons, though the mechanism is unknown (Pereira et al. 2000). Granzyme B, instead of directly cleaving the normal target procaspase 3, instead cleaves the IE protein ICP4 (Knickelbein et al. 2009). ICP4 has been shown to be necessary for efficient reactivation of the virus, and cleavage by granzyme B has been shown to be a noncytolytic mechanism by which CTLs can react to viruses and virus-infected neurons and thereby control reactivation.

Additionally, interferon-γ produced by the CTLs plays a pivotal role in preventing reactivation. Decman et al. (2005) previously demonstrated that interferon-γ is capable of reducing expression of ICP0 promoters, possibly by global regulation of transcription factors. Carr et al. (2009) showed that transgenic expression of interferon-γ in the trigeminal ganglia reduced HSV-1 reactivation in mouse models when exposed to ultraviolet light. Another important role of interferon-γ centers on increasing the expression of the heavy and light chain of MHC class I molecules in addition to chaperones and other proteins that help assemble the peptide–MHC class I complex (Wallach et al. 1982). Interferon-γ induces expression of MHC class 1 in neurons which normally lack MHC class 1 expression (Wallach et al. 1982). Interestingly, Chentoufi et al. (2011) showed that expression of LAT in neuroblastoma cell lines also increased expression of MHC class I molecules. To counter increased presentation from upregulated MHC class I expression, the HSV-1 IE protein ICP47 interacts with the TAP proteins and prevents import of antigenic peptides

into the endoplasmic reticulum (Oosten et al. 2007). This has been shown to inhibit display of HSV-1 antigen on the surface of the neurons. One study has suggested that ICP47 activity is required for HSV-1 to infect neuronal cells (Burgos et al. 2006). An interesting effect of the mouse model of HSV-1 latency has involved the fact that ICP47 from human viruses does not efficiently interact with TAP from the mouse, with the effect being that HSV peptides are efficiently displayed. This may be the reason that mice with latent HSV infection, unlike humans, do not exhibit spontaneous reactivations of virus (Feldman et al. 2002). To investigate the effect of TAP and MHC Class 1 inhibition by viral proteins on reactivation, Orr et al. (2008) generated recombinant HSV-1 virus which expressed the murine cytomegalovirus gene m152 (MCMV m152). The MCMV m152 gene has been shown to be capable of inhibiting MHC class 1 export and decreases expression on the cell surface (Tomas et al., 2010). When mice were infected with the recombinant HSV-1, they exhibited increased virus reactivation from latency. However, this model does not reflect spontaneous reactivation as it still relies on UV light to stimulate reactivation.

1.5. Latent HSV-1 modulation of apoptosis

Apoptosis has been shown to be the preferred method that the body uses to eliminate viral infections. This pathway leads to programmed cell death characterized by DNA fragmentation and membrane blebbing, which will be taken up by local immune phagocytes. By inducing cell

death, infected cells and the viruses contained within are removed. Most cells removed via apoptosis are capable of being replaced by stem cell precursors, and thus elimination of the infected host cell is a favorable mechanism of limiting viral disease. This process in combination with the antiviral state induced by interferon signaling has been shown to be the primary method of limiting viral replication in the body and clearing viral infections (Benedict et al. 2002).

Apoptosis can be triggered in multiple ways that work through a set of proteases called caspases. These mediators of cell death are synthesized as inactive precursors that must in turn be cleaved in order to be activated. Proapoptotic stimuli lead to cleavage of procaspase to caspase, which then proceed to cleave their own substrates in turn. The extrinsic pathway can be activated by a variety of external factors such as cytokines, toxins, or ligand binding to death receptors on the cell surface. The intrinsic apoptosis pathway is triggered by cell stress factors within, such as viral proteins, DNA damage, and oxidative stress, leading to dimerization of proapoptotic molecules on the surface of the mitochondria. This forms a dimer through which cytochrome C has been shown to be released into the cytoplasm. Cytoplasmic cytochrome C associates with Apaf1 to activate procaspase 9. Activated caspase 9 complexes with Apaf1 and cytochrome C to form the apoptosome, which efficiently cleaves and activates procaspases 3 and 7. Both of these pathways activate a different set of caspases, which proceed to clean downstream

targets. These pathways converge on the activation of procaspase 3, which can also be cleaved directly from its precursor from CTL-released granzyme B. Activated caspase 3 leads to chromatin condensation, DNA fragmentation, and the membrane blebbing characteristic of apoptosis, which has been reviewed previously (Elmore 2007; Hengartner 2000).

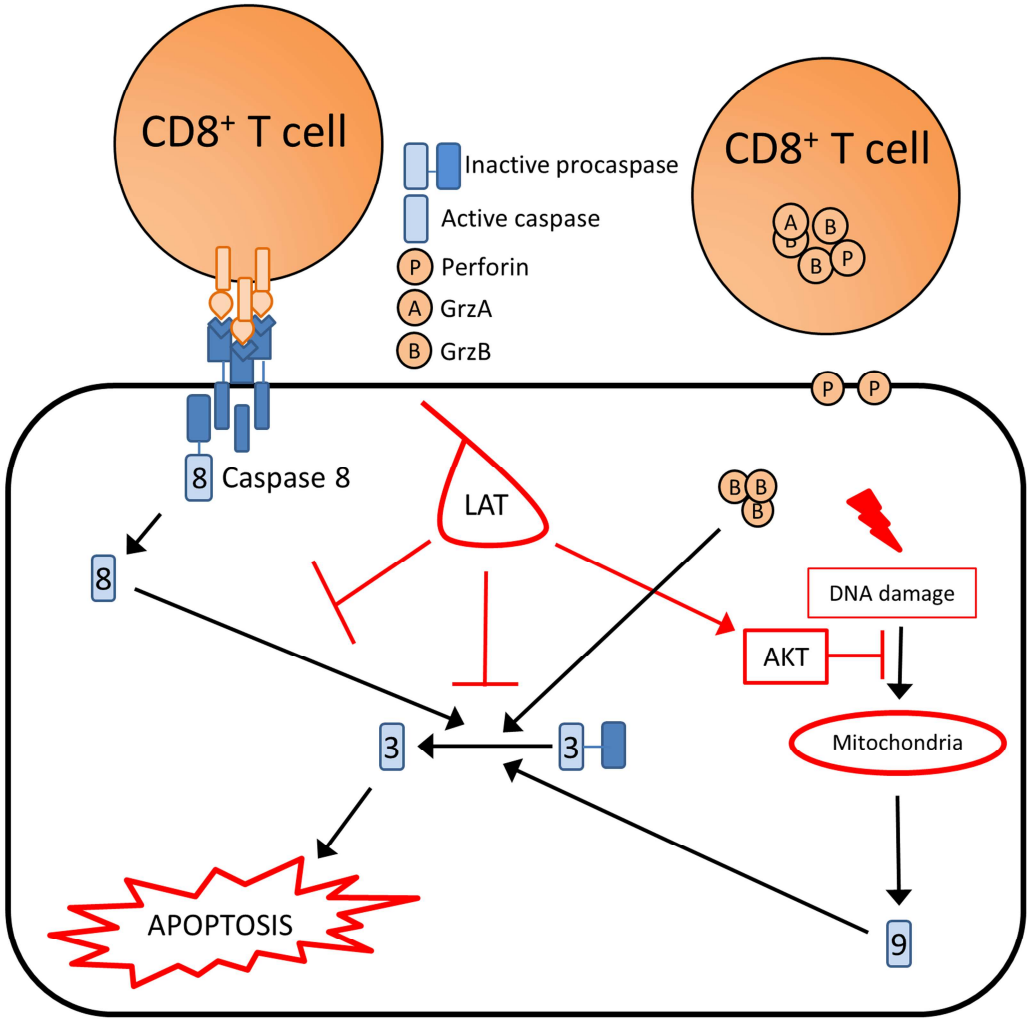
Herpesviruses produce numerous antiapoptotic factors aimed at delaying or preventing apoptosis to allow for maximal number of infectious virions to be produced. Studies performed with specific null mutants have revealed that ICP4 (Leopardi and Roizman, 1996), ICP27, Us3 (Aubert et al., 2006; Jerome et al., 1999), gD (Sciortino et al., 2008) , gJ (Jerome et al., 2001), and LAT all have antiapoptotic functions in vitro, though the mechanism is not fully understood for all of them. Recent data has suggested that cathepsins are involved in ICP4- and Us3-mediated inhibition of apoptosis (Peri et al., 2011). Antiapoptotic gene transcription in productively infected epithelial cells allows the virus to evade apoptosis, and the cell dies from damage induced by viral replication. The LAT intron is the predominant transcript that can be recovered from latently infected neurons and conveys resistance to apoptosis. Although not absolutely required for establishment of latency, it does play an important role in maintenance, and reactivation of latency, in addition to the antiapoptotic effects associated with these RNAs (Bloom 2004).

The antiapoptotic effects derived from LAT in latently infected neurons have not been fully elucidated. The unstable 8.3-kb primary LAT

has been shown to be processed to form a stable 2.0- or 1.5-kb intron (Wagner et al., 1988). HSV-1 LAT null mutants confer decreased protection from cold shock–induced apoptosis (Carpenter et al. 2007). Using specific HSV-1 deletion mutants, Branco and Fraser (2005) localized the antiapoptotic function in the first 1.5-kb region following the LAT promoter. Granzyme B–mediated apoptosis via direct cleavage of procaspase 3 was inhibited by LAT expression (Jiang et al. 2011). LAT has also been shown to inhibit caspase-8-dependent apoptosis induced through death receptor binding (Jin et al. 2010). LAT upregulated expression of the prosurvival protein AKT, which prevented the intrinsic apoptotic pathways initiated within cells (Figure1.2.) (Li et al. 2010; Manning and Cantley 2007).

Figure 1.2. Expression of latency-associated transcript (LAT) inhibits extrinsic and intrinsic apoptotic pathways. The extrinsic pathway involves proapoptotic triggers provided by activated immune cells. Virus-specific $CD8^+$ T cells induce apoptosis by death receptor binding. CD95 ligand binding causes the formation of the death-induced silencing complex, which binds and activates procaspase 8. Activated caspase 8 activates procaspase 3, which leads to apoptosis. LAT expression is capable of inhibiting the caspase-8-dependent pathway, but the mechanism is unknown. $CD8^+$ T cells releasing preformed granules induce apoptosis by direct cleavage of procaspase 3. Perforin forms a pore on the surface of target cells and allows entry of granzyme B (GrzB). Granzyme B cleaves and activates procaspase 3, leading to apoptosis. LAT inhibits this pathway by inhibiting granzyme B from activating procaspase 3. The intrinsic apoptotic pathway is initiated by dimerization of proapoptotic molecules on the surface of the mitochondria. Released cytochrome C associates with APAF1 and activates procaspase 9. Activated caspase 9 activates procaspase 3. Expression of LAT activates AKT, which prevents the accumulation of proapoptotic molecules.

Figure 1.2.


CD8+ T cell
Inactive procaspase
Active caspase
Perforin
GrzA
GrzB
CD8+ T cell
A
B
B
P
P
P
8
Caspase 8
LAT
B
B
B
8
DNA damage
AKT
3
3
Mitochondria
APOPTOSIS
9

The LAT sequence does contain eight putative open reading frames (ORF) that could encode for antiapoptotic proteins; however, HSV-1 LAT-associated proteins have not been isolated (Drolet et al., 1998). There is circumstantial evidence for LAT-associated proteins within latent infections. Henderson et al. (2009) generated synthetic peptides from the LAT ORF sequences and injected these into rabbits to generate antisera against the peptides. Antisera from two of the peptide sequences bound to protein with neuronal cell cultures. Additionally, immunohistochemistry staining of latently infected trigeminal neurons was also positive utilizing these antisera. However, the role of LAT-associated proteins is still controversial. The structure, abundance, and activity of these proteins have not been thoroughly investigated; it has been proposed that it may be possible that they play a role in inhibiting apoptosis of infected neurons but there will need to be further studies to establish this functional connectivity.

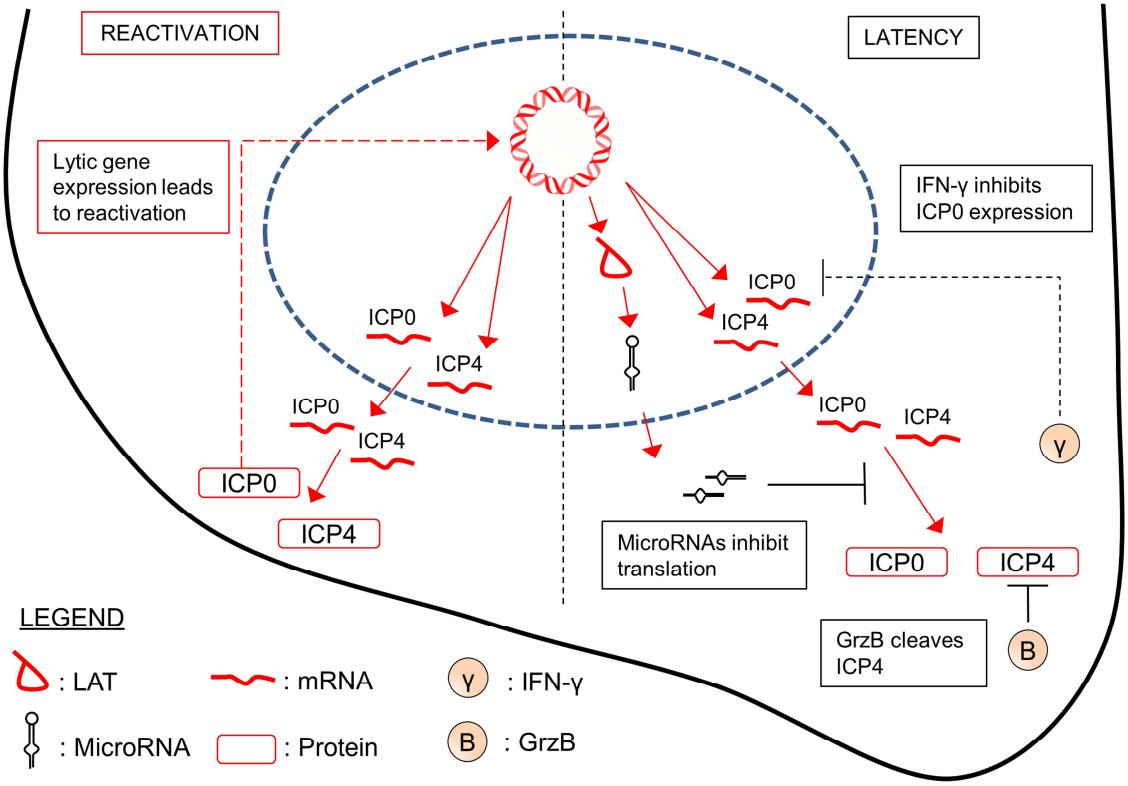
An additional mechanism of antiapoptosis activity could lie in the encoding of viral microRNA within the LAT (Jurak et al. 2012). Because of the compact nature of viral genomes in which viruses must package everything they need for productive infection, the encoding of microRNAs in nontranscribed regions and untranslated regions of exons can provide important regulatory elements at the transcriptional level (Boss and Renne 2010; Gottwein and Cullen 2008; Skalsky and Cullen 2010). Using deep sequencing analysis of postmortem latently infected trigeminal ganglia;

Umbach et al. (2009) identified seven miRNAs encoded by the HSV-1 LAT, all of them contained within the unstable 6.3-kb exon. These miRNAs have been recovered from postmortem human trigeminal tissues, demonstrating their presence in human HSV-1 infections. The targets for all of the miRNAs have not yet been identified, but miR-H2 has been demonstrated to target ICP0 and miR-H6 targets ICP4 (Umbach et al., 2008). Recently it has been shown that mutation of these miRNAs decreases replication of HSV-1 within neurons but has no effect on replication within fibroblasts (Flores et al., 2013). ICP0 and ICP4 are potent activators of the β genes and are necessary for reactivation. Additionally, ICP0 expression can induce apoptosis through damage of cellular structures (Sanfilippo and Blaho 2006). The LAT-encoded miRNAs are contained outside of the vital first 1.5-kb region, which has been shown to be necessary for protection from apoptosis, so it is unlikely these are the main mechanism involved in the inhibition of apoptosis. However, by regulating viral gene transcription, miRNAs can help maintain the virus in a latent state within neurons and prevent expression of proapoptotic genes. Similar to the miRNAs, LAT encodes two small RNAs (sRNAs). LAT sRNA2 has been shown to hybridize to the ICP4 transcript and reduce its expression. Additionally, sRNA1 and sRNA2 act synergistically to inhibit apoptosis induced by cold shock (Shen et al. 2009). The mechanisms by which LAT prevents apoptosis are not yet known, but LAT

appears to promote the latent state through viral gene regulation by miRNAs and sRNAs encoded within LAT (Figure 1.3.).

Figure 1.3. Small noncoding RNAs in the latency-associated transcript (LAT) gene promote latency. Low-level expression of lytic genes *ICP0* and *ICP4* could lead to spontaneous reaction. The LAT gene encodes two different small noncoding RNA species that prevent low-level *ICP0* and *ICP4* expression. The LAT gene encodes seven microRNAs (miRNAs) that have been confirmed in vivo. Of these, two miRNAs, miR-H2 and miR-H6, have confirmed inhibitory effects on ICP0 and ICP4 protein expression; miR-H2 acts on ICP0 and miR-H6 acts on ICP4. By preventing translation of lytic genes required for reactivation, small noncoding RNAs derived from LAT promote the maintenance of latency. Granzyme B (GrzB) and interferon-γ (IFN- γ) released from $CD8^+$ T lymphocytes also contributes to the maintenance of latency. GrzB cleaves the ICP4 protein and IFN-γ inhibits expression of ICP0.

Figure 1.3.


REACTIVATION
LATENCY
Lytic gene expression leads to reactivation
IFN-γ inhibits ICP0 expression
ICP0
ICP4
MicroRNAs inhibit translation
GrzB cleaves ICP4
γ
B
LEGEND
: LAT
: mRNA
: IFN-γ
: MicroRNA
: Protein
: GrzB

In contrast to latently infected neurons, productively infected cells in the periphery undergo cell death. Peripheral epithelial cells are susceptible to lytic infection leading to necrosis during productive HSV-1 replication. Other cell types including monocytes and DCs can be induced to initiate apoptosis from both intrinsic proapoptotic stimuli from the virus and from exogenous signals from immune cells (Mastino et al. 1997; Peri et al. 2011). In order to definitively identify which cells are susceptible to apoptosis, Esaki et al. (2010) exposed mice to HSV-1 and HSV-2 using three different inoculation techniques and collected various tissues for HSV-1 antigen staining and apoptotic markers. They found that cells such as those of the corneal epithelium and neurons in the CNS are sensitive to induction of apoptosis when infected by HSV-1. They also found that cells within the trigeminal ganglia, although susceptible to HSV-1 infection, were not apoptotic. These results fit well with previous experiments that have shown that corneal epithelia cells from both animal models and humans undergo apoptosis when infected with HSV-1 (Stuart et al. 2004). However, this has been challenged by Miles et al. (2007), who demonstrated that although apoptosis is induced during productive infection of corneal epithelial cells, HSV-1 has been shown to be capable of inhibiting the later stages of apoptosis.

1.6. Maintenance of latency requires careful balance of factors

During neuronal latency, a careful balance has been shown to be established to maintain the virus in the latent state. The contributors to this balance include the neuronal environment, LAT, and the $CD8^+$ T cells surrounding the neurons. During latency, the LAT intron has been shown to be the predominant transcript that is made from the viral genome (Wagner et al., 1988). There is evidence, however, of low-level lytic gene expression despite the presence of repressive chromatin modifications (Ramachandran et al., 2010). It has been suggested that in neuronal cultures, low-level gene expression is required for the production of VP16, a γ gene. Once VP16 has been made, the VP16 transcription complex can form and bind to the lytic gene promoters, leading to production of infectious virions (Kim et al. 2012). It is this low-level gene expression that was detected by surrounding $CD8^+$ T cells that release interferon-γ and granzyme B, mediators capable of inhibiting lytic gene expression. Disruption of $CD8^+$ T-cell inhibition of viral lytic gene expression could lead to reactivation from initial ganglionic neuron steady state low-level gene expression. Some of the specific stimuli associated with reactivation include menstruation in women and psychological and physiological stress. Treatment with medroxyprogesterone acetate, a synthetic analogue of the female sex hormone progesterone, released during ovulation, has been shown to be capable of reducing $CD8^+$ T-cell levels in the trigeminal ganglia of latently infected mice (Himmelein et al. 2011).

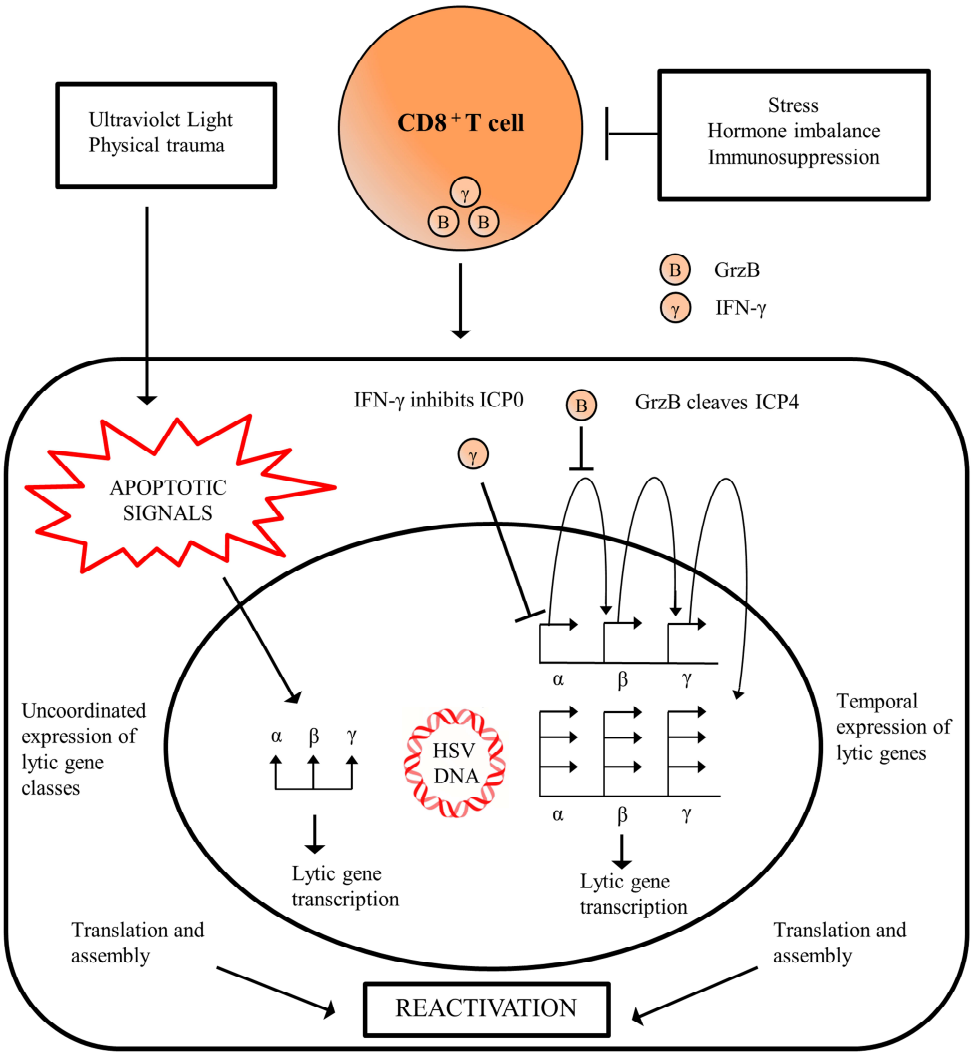
Psychological and physiological stress have also resulted in a reduction of $CD8^+$ T cells in the trigeminal ganglia and reduced capacity to secrete interferon-γ (Freeman et al. 2008). The stress response is mediated through release of the glucocorticoid cortisol, which has been shown to reduce interferon-γ expression in T cells (Curtin et al. 2009).

The mechanism by which $CD8^+$ T cells inhibit lytic gene expression has been shown to be through the release of interferon-γ and granzymes A and B. Release of granzyme B has been shown to be a primary method through which $CD8^+$ T cells induce apoptosis in virus-infected cells. However, neurons harboring latent HSV-1 are protected from granzyme B–induced apoptosis (Knickelbein et al., 2009). The exact mechanisms of this protection have not been thoroughly explored, but it appears that LAT is critical for inhibiting apoptosis in infected neurons (Jiang et al., 2011). The inhibition of apoptosis in latently infected neurons reflects the other side of the careful balance that allows the latent infection to be maintained. Disruption of this balance is another mechanism through which external stimuli can potentially induce reactivation. If neurons that harbor the latent viral genome are induced to undergo apoptosis, the virus reactivates and releases infectious virions (Figure 1.4). The pathway through which apoptotic signals initiate reactivation is unknown. This is potentially a mechanism by which physical damage to neurons induces reactivation. Additionally, apoptotic signals can be a mechanism by which ultraviolet (UV) light could induce reactivation from latency. The axons of

innervating neurons extend into the periphery and are in proximity to the surface of the skin, and could be susceptible to UV light exposure. UV light can induce apoptosis in cells through either direct damage of DNA or by production of reactive oxygen species (ROS) (Kulms et al., 2002). Mitochondria in neurons are uniformly distributed throughout the neuron, including the axon (Hollenback 1996). UV light exposure can induce production of ROS in mitochondria residing near the axonal terminal, which could lead to apoptotic signaling. ROS has been associated with reducing expression and levels of Bcl-2 and inhibiting mammalian target of rapamycin (Alexander et al. 2010), responses that have been shown to induce reactivation (Kobayashi et al. 2012).

Figure 1.4. Pathways of stimuli induced reactivation. Low-level temporal expression of lytic genes can lead to production of the transcription factor complex and efficient temporal expression of lytic genes. This low-level expression has been shown to be inhibited by factors released from surrounding $CD8^+$ T cells. Interferon-γ (IFN-γ) inhibits ICP0 expression and granzyme B (GrzB) has been shown to cleave the ICP4 protein required for efficient expression of lytic genes. Stimuli such as hormone imbalance and stress inhibit $CD8^+$ T-cell activity, which allows low-level lytic gene expression to continue. Lytic gene expression leads to assembly of viral components, release, and reactivation (right). Alternatively, stimuli such as UV light and physical trauma initiate gene transcription through apoptotic signals. Apoptotic signaling leads to uncoordinated expression of lytic genes and production of new infectious viruses and reactivation (left).

Figure 1.4.


Ultraviolct Light
Physical trauma
CD8+ T cell
Stress
Hormone imbalance
Immunosuppression
GrzB
IFN-γ
IFN-γ inhibits ICP0
GrzB cleaves ICP4
APOPTOTIC
SIGNALS
Uncoordinated
expression of
lytic gene
classes
HSV
DNA
Temporal
expression of
lytic genes
Lytic gene
transcription
Lytic gene
transcription
Translation and
assembly
Translation and
assembly
REACTIVATION

Studies by Du et al. (2012) have previously demonstrated an interesting feature of the reactivation process when neurons are treated with chemical agents that reduce the levels of Bcl-2 and Bcl-xl. Interestingly, during this process the expression of the viral genome did not proceed according to the ordinary temporal pattern observed in primary infection. In contrast to sequential expression of the α, β, and γ genes, all gene classes were expressed at the same time upon induction of apoptosis. This is a mechanism by which the virus can rapidly produce new virions in response to signals that indicate that the cell in which it is residing is no longer viable. Rapid virus assembly and escape allows the virus to infect peripheral epithelial and neuronal cells to remain in the host while being transmitted to other individuals during recurrent disease.

1.7. Tissue resident memory T cells and HSV-1 infection

It is possible that the $CD8^+$ T cells surrounding latently infected ganglia belong to a novel subset of memory T cells recently described called tissue resident memory T cells (T_{RM}). Following antigenic exposure, two distinct populations of memory T cells are normally found. Central memory T (T_{CM}) cells are retained in secondary lymphoid organs and have the capacity to rapidly proliferate upon reexposure to antigen. These are characterized by specific cell surface markers $CD44^+$ $CCR7^+$ $CD62L^+$. In contrast to central memory T cells, effector memory T (T_{EM}) cells are $CD44^+$ $CCR7^-$ $CD62L^-$ (Harty and Badovinac 2008). The T_{EM} cells circulate through the blood and numerous tissues, sampling the environment for

their specific antigen. These cells are already differentiated and are capable of immediate effector functions and can begin clearing infections without the need to proliferate and differentiate. Memory T-cell responses provide fast responses to reinfection, as opposed to how they respond to novel antigens, but they rely on the specific antigen to be delivered to secondary lymphoid organs (for T_{CM} cells) or for the antigen-specific T_{EM} cell to be present at the site of reinfection at the same time. A population of memory T cells that reside long-term at the site of initial infection would be capable of immediate response to antigenic exposure in the site where exposure is likely to occur.

Recent evidence supports the notion of a distinct population of memory T cells that are retained in the tissue of initial exposure. T_{RM} cells have been found in numerous tissues in the body, including brain parenchyma (Hawke et al. 1998), skin (Gebhardt et al. 2009), genital mucosa (Tang and Rosenthal 2010), gut ileum (Masopust et al. 2010), salivary glands (Casey et al. 2012), and the dorsal root ganglia (DRG) (Gebhardt et al. 2009). Although surface marker characterization has not been done with T_{RM} from all these locations, some distinctive phenotypic markers have been identified. T_{RM} cells are $CD44^+$ $CCR7^-$ $CD62L^-$ $CD69^+$ $CD103^+$ (Wakim et al. 2012). CD103 has not been found in all tissue sources of T_{RM} cells, but it does appear in the majority of T_{RM} cells isolated. CD103 is the α chain of the α4β7 integrin receptor for E-cadherin normally expressed in epithelial cells (Cepek et al., 1994). An important

characteristic of T_{RM} cells is that they do not migrate through the periphery or enter into the secondary lymphoid organs; expression of CD103 likely allows T_{RM} cells to be retained in tissue compartments. T_{RM} cells have been shown to be capable of persisting for extended periods in these peripheral tissue compartments without replenishment from the circulating pool of $CD8^+$ T cells owing to low homeostatic turnover (Gebhardt et al. 2009; Masopust et al. 2006). An interesting distinction of T_{RM} cells is their apparent ability to survive without IL-15 signaling, even in the absence of antigenic exposure (Wakim et al. 2010). The combination of CD103 expression and long-term residence in tissues where antigen was initially encountered distinguishes T_{RM} cells from the established memory T cells.

With the recent characterization of T_{RM} cells, it is important to analyze what other T-cell populations might belong to this novel subclass. In latent HSV-1 infection, $CD8^+$ T cells surrounding infected neurons in the trigeminal ganglia persist for the lifetime of the organism and are vital in maintaining the virus in a latent state (Hoshino et al. 2007; Liu et al. 2000). This is accomplished through expression of effector molecules, and therefore HSV-1-specific $CD8^+$ T cells have an activated phenotype akin to effector memory cells. Activated $CD8^+$ T cells have the markers CD69, CD44, CD25, and CD49d expressed on their surface. They downregulate homing receptors CD62L and CCR7 (Barrat et al. 1995; Lynch et al. 1989) and the markers of naïve $CD8^+$ T cells CD27 and CD28 (Verjans et al. 2007). Additionally, the $CD8^+$ T cells in the trigeminal ganglia express

granzymes A and B and interferon-γ. Like T_{RM} cells, IL-15 is not required to maintain the population within the trigeminal ganglia (Sheridan et al. 2006). The α4β7 integrin CD103 is a marker used to consistently identify T_{RM} cells, but no experiments have addressed whether CD103 is present on the surface of trigeminal ganglia resident $CD8^+$ T cells.

Additionally, the migration pattern of $CD8^+$ T cells differs extensively from patterns observed with T_{EM} cells. Principally, $CD8^+$ T cells never leave the trigeminal ganglia once they have infiltrated and surrounded latently infected neurons (Khanna et al. 2003). Whereas T_{EM} cells migrate through numerous tissues and can participate in peripheral immune responses to antigen reexposure, $CD8^+$ T cells reside in the trigeminal ganglia for the lifetime of the host. Himmelein et al. (2011) provided evidence of the peculiar homing pattern of the HSV-1-specific $CD8^+$ T cells retained in the trigeminal. Following treatment with corticosteroids and challenged with restraining stress, stimuli proven to reduce $CD8^+$ T-cell levels in the trigeminal ganglia (Elftman et al. 2010; Freeman et al. 2008), the population of $CD8^+$ T cells in the trigeminal ganglia is quickly reestablished (within 4 days). Importantly, the trigeminal population is not supplemented with $CD8^+$ T cells from the circulation, nor is it due to proliferation of residual $CD8^+$ T cells in the trigeminal ganglia immediately after the treatment. These results imply that the $CD8^+$ T cells that left the trigeminal ganglia are capable of homing back to their original location. $CD8^+$ T cells in the periphery are incapable of infiltrating the

trigeminal ganglia, which prevents supplementation or replacement of the population from the circulating pool of $CD8^+$ T cells. The same population is not maintained based on the proliferation of resident $CD8^+$ T cells; instead, these cells have very low homeostatic turnover.

Currently available information on trigeminal resident $CD8^+$ T-cell surface markers indicates they could fall into either the T_{EM} class or the recently discovered T_{RM} class (Table 1.1.). The migration dynamics are the primary evidence that the trigeminal ganglia resident $CD8^+$ T cells could be T_{RM} cells instead of T_{EM} cells. These T cells are retained in the same tissue compartment for the lifetime of the host and home back to the same location if dislodged. These cells survive in the tissue compartment without being replenished from circulating pools of $CD8^+$ T cells, have low homeostatic turnover, and do not require IL-15 for survival. Further characterization of the cell surface phenotype of this cell population is needed to definitively classify them as being either T_{RM} or T_{EM} cells.

Table 1.1.

Table 1. Phenotypic markers of memory $CD8^+$ T cells

	Memory T-cell subtypes			
	T_{CM}	T_{EM}	T_{RM}	Trigeminal ganglia resident $CD8^+$ T
CD44	+	+	+	+
CD69	-	-	+	+
CD103	-	-	+	
Tissue retention	+	-	+	+
Effector function	-	+	+	+

T_{CM}, central memory T cells; T_{EM}, effector memory T cells; T_{RM}, tissue resident memory T cells.

1.8. T_{RM} cells and immunity to viruses

Gebhardt et al. (2009) studied the response of T_{RM} cells to reinfection extensively in the context of HSV-1 skin infection. Following flank infection with HSV-1, they found an accumulation of adoptively transferred HSV-1-specific $CD8^+$ T cells ipsilateral to the infection site but not on the control infection site. These cells were located in the epithelial layer of skin; had a surface marker phenotype $CD69^+$ $VLA1^+$ $CD103^+$ $CD62L^-$ $CD122^-$; did not migrate; and had a slow homeostatic turnover. Importantly, when challenged with virus following a previous exposure and clearance of virus, the skin flanks with residing T_{RM} cells had much better protection than the control flank. This enhanced protection was shown to be T-cell dependent, with contributions from both $CD4^+$ T and $CD8^+$ T cells. Eliminating $CD4^+$ T cells had a significant effect on clearance, but when provided with excess $CD8^+$ T cells, they were capable of clearance without the help of $CD4^+$ T cells.

It is important to note that the results described above involved reexposure to exogenous virus and cannot be directly related to skin T_{RM} cells responding to reactivation of virus. Mouse models of latent HSV-1 infection have very rare episodes of spontaneous reactivation (Gebhardt and Halford 2005), and thus chemical agents or stressful stimuli, which often decrease trigeminal ganglia resident $CD8^+$ T cells, are required for reactivation. It is unknown what effect these agents might have on T_{RM} cells in the skin and whether they have any effect on virus clearance.

Humans do experience spontaneous reactivation or reactivation as a result of certain exogenous agents. Reactivation of latent virus can result in asymptomatic shedding of virus or may cause formation of recrudescent lesions in the same peripheral location where the primary infection occurred. Recurrent disease provides circumstantial evidence that if T_{RM} cells exist in humans, they either do not prevent reinfection where they reside or the agent responsible for reactivation also has an effect on peripheral T_{RM} cells.

Rates of reactivation are inversely correlated with the number of $CD8^+$ T cells infiltrating the trigeminal ganglia and have been directly correlated with the number of latently infected neurons (Hoshino et al. 2007). Initial hopes that HSV-1-specific $CD8^+$ T cells could be expanded in vitro and then returned to the host as a method to reduce reactivation rates have failed because adopted $CD8^+$ T cells cannot access the trigeminal compartment where latently infected neurons reside (Himmelein et al. 2011). It is possible that critical comparisons of circulating and resident ganglionic $CD8^+$ T cells will reveal new information that will make it possible to adoptively transfer T cells into the trigeminal compartment in order to reduce reactivation and recurrent disease. This method would not be capable of eliminating the latent infection, but a robust immune presence is capable of limiting virus reactivation from latency.

Despite decades of research on HSV-1, there is still no effective vaccine that can prevent virus infection or control reactivation (Dervillez et

al., 2012). Following primary infection in the periphery, released virions encounter and infect innervating sensory neurons. The virus attaches to the axonal termini and releases the capsid and tegument proteins. The capsid and possibly one or more of the tegument proteins migrate retrograde towards the neuronal soma where the DNA is uncoated, injected into the nucleus. Viral latency is established very early during the course of the primary infection in at least a subset of infected neurons. During reactivation, capsids are assembled in the nucleus and then transported across the nuclear membrane as enveloped particles. Once in the neuronal cytoplasm they are thought to travel, as enveloped or possibly nonenveloped particles, anterograde towards the axonal terminal region with infectious virions released into the periphery to infect other cells as cell-free virus or possibly by mechanisms involving cell-to-cell transfer of neuronal membrane-associated virus particles (Smith, 2012). This implies that during the migration of virus from the latent reservoir to target cells, few locations exist where neutralizing antibodies can act upon the virus before it is exposed to target cells. There is a robust immune response leading to expanded $CD8^+$ T-cell populations surrounding infected cells in the trigeminal, but these cells are incapable of eliminating the virus, only of limiting viral reactivation. This means that the primary location where a vaccine must be effective is the site of primary infection.

Mackay et al. (2012) revealed that expanded HSV-specific T_{RM} cells in the periphery are capable of limiting disease from reexposure to HSV-1.

These experiments suggest an exciting new direction for developing a vaccine to prevent new infections or limiting the severity of recurrent disease. Shin and Iwasaki (2012) adapted this concept and hypothesized that a “prime and pull” technique can generate large numbers of virus-specific $CD8^+$ T cells in the periphery, where they can stop or limit damage from infection. The “prime” is composed of a subcutaneous injection of thymidine kinase (TK^-) HSV-2 virus, capable of activating and proliferating adoptively transferred $CD8^+$ T cells. The “pull” is accomplished by topical treatment of CXCL9 and CXCL10 chemokines applied to the vaginal cavity. The chemokine treatment attracted many more $CD8^+$ T cells to the vagina compared with immunization without chemokine treatment. Treatment provided after the central pool of $CD8^+$ T cells had been activated successfully recruited and maintained T cells in the genital tract for up to 12 weeks after treatment. When challenged with a lethal dose of wild-type HSV-2, mice receiving the immunization and chemokine treatment suffered less weight loss, did not develop clinical symptoms, and had a 100% survival rate. Importantly, viral titers found in the DRG following WT infection were significantly decreased compared with immunization alone. Because reactivation rates are directly correlated with latent viral load, a therapeutic intervention that lowers the initial viral infection of the DRG would be beneficial. This novel vaccine treatment appears to have successfully lowered the viral burden in the DRG,

decreased the number of latent viral genomes, and would likely significantly decrease reactivation and recurrent disease in humans.

1.9. Conclusions

HSV-1 represents a persistent human pathogen that resides in infected hosts for their lifetime. Clearance of the primary infection follows establishment of the latent infection within the ganglionic clusters of innervating sensory neurons. Reactivation of the virus is controlled at least in part by $CD8^+$ T cells that surround latently infected neurons. An important feature of these cells is their long-term residence in the neuronal tissue compartment. HSV-1-specific $CD8^+$ T cells can remain in the trigeminal ganglia for the lifetime of the host, do not rely on replenishment from circulating $CD8^+$ T cells, and have the capability to home back to the trigeminal ganglia if displaced. These behaviors distinguish the resident ganglionic $CD8^+$ T cells from the traditional $CD8^+$ T-cell memory subsets.

Recently a new class of $CD8^+$ memory T cells that reside in peripheral tissue compartments has been characterized. The phenotypic characteristics of the T_{RM} cell are similar to those of the trigeminal ganglia resident $CD8^+$ T cells that arise in response to HSV-1 infections. Both cell types exist in peripheral tissue compartments for long periods, have low homeostatic turnover, are not replenished from the circulating $CD8^+$ T cell pool, and express the effector molecules interferon-γ and granzyme B. The CD103 integrin receptor is a surface marker used to identify T_{RM} cells in peripheral tissues, but its expression has not been evaluated in

trigeminal ganglia resident $CD8^+$ T cells. Identification of HSV-1-specific $CD8^+$ T cells as T_{RM} cells can lead to exploration of new approaches towards reducing disease in humans. Discovery of the factors associated with homing of the trigeminal resident $CD8^+$ T cells back to the peripheral nervous system compartment may lead to new ways of bolstering the $CD8^+$ T-cell levels in the trigeminal ganglia to lower reactivation rates. New vaccine approaches designed to attract and retain T_{RM} cells in peripheral tissue locations in response to immunization could lower viral burden on peripheral nervous system following exposure to virus.

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Chapter II

Modeling HSV-1 infection orofacial epithelial surface in the mouse

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Kevin Egan conceptualized experiments, performed experiments, collected and analyzed data and wrote the chapter. Alexander Allen collected data. Drs. Stephen Jennings and Brian Wigdahl participated in the intellectual development, conceptualized experiments and critically evaluated all aspects of the chapter. Dr. Stephen Jennings is supported by developmental funds provided by the Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine

2.1. ABSTRACT

The lip scarification model of herpes simplex virus type 1 (HSV-1) infection can be used to study acute infection in the epithelial anatomical surfaces of the mouse lip and the establishment of viral latency. Adult mice were infected with HSV-1 McKrae and during the acute phase of infection, epithelial and ganglionic tissues were harvested and analyzed. Clinical presentation of classical open sores on the epithelial surface of the lips of infected mice was observed. We further defined the histopathology and immune infiltration of the lower lip during the formation and resolution of the clinical lesions. Finally, the kinetics of virus replication and transport of viral genomes to the trigeminal ganglia were established. With the virological and immunological events of acute infection defined, the HSV-1 lip scarification model can now be used to continue studies focused on primary viral infection, invasion of the peripheral nervous system, establishment of virus latency, and the viral and host factors involved in modulating the latent state and the reactivation process.

2.2. INTRODUCTION

Herpes simplex virus type 1 (HSV-1) is a pervasive human pathogen which is predicted to infect up to 90% of adults worldwide (Xu et al., 2006). The majority of new human infections occur in the oral mucosa directly adjacent epithelial surfaces during childhood. Following replication in the periphery, the virus establishes a latent infection in the trigeminal ganglia (TG) which persists for the lifetime of the host. Periodic reactivations cause recrudescent lesions in orofacial tissue termed herpes labialis or cold sores. Infected persons are capable of shedding the virus asymptomatically (Schulte et al., 2014), though shedding of virus increases in those suffering symptomatic reactivations (Mark et al., 2008). Immunocompromised individuals who cannot control replication of the virus during acute and latent stages are at greater risk for more severe systemic disease with potential invasion of the central nervous system (CNS) and the development of encephalitis (Tan et al., 2012).

Initial infection of the lip comes from a compromise in the epithelial barrier, providing the virus with access to the underlying keratinocytes. Infection in the epithelia causes localized pathology within the lip and activation of the immune response. Infected epithelial cells display characteristic viral inclusions bodies including multinucleated cell bodies, keratinocytes with steel grey nuclei, orthokeratosis, and chromatin margination (Leinweber et al., 2006). Infection of the epithelial area surrounding hair follicles leads to a condition called herpes folliculitis (Böer et al., 2006). The histopathology of herpes folliculitis can differ from other HSV-1 infections by a lack of multinucleated cell bodies and steel grey

nuclei, however, dense immune infiltrates surrounding hair follicles and necrotic keratinocytes can be observed. After establishing infection in the epithelia, the virus gains access to innervating sensory neurons from the trigeminal ganglia. Retrograde transport from the epithelia to the trigeminal ganglia is followed by establishment of viral latency for the lifetime of the host. After any of a number of physiological stimuli, reactivation of viral gene expression and replication occurs, the virus travels in an anterograde direction down the same infected sensory neurons, with viral infectivity transferred to neighboring cells in the periphery to establish a new infection in the epithelia in close anatomical proximity to the primary infection and termed recurrent infection/disease.

There is a dense accumulation of sensory nerve endings in the skin of the lower lip. These nerve endings convey information on mechanical, thermal, chemical, touch, and pain stimuli through A-beta, A-delta, C type fibers (Yosipovitch and Papoiu, 2012). The sensory nerves together form the mental nerve which is responsible for sensory perception in the lower lip and chin. The mental nerve inserts into the mental foramen and combines with other sensory branches to form the inferior alveolar nerve. The inferior alveolar nerve and lingual nerve together form the mandibular branch of the trigeminal ganglia (Wilson-Pauwels, 2001). These neuronal cells are the likely major targets of HSV-1 infection in the lip epithelium during the course of primary infection and subsequent reactivation events. During reactivation, trigeminal neuralgia has been reported to occur in the peripheral regions where reactivation is going to occur. The neuralgia is likely due to irritation of the sensory neurons during HSV-

1 reactivation and can be an early symptomatic sign of recurrent disease (Gonzales, 1992).

The immune system plays a crucial role in restricting viral replication to the periphery, resolving the primary infection, and maintaining the virus in latency as previously reviewed (Egan et al., 2013). An interesting facet of HSV-1 biology is that the double-stranded RNA (dsRNA) sensory Toll Like Receptor-3 (TLR-3) appears to be important with respect to preventing the virus from gaining access to the CNS. Patients with mutations in the TLR-3 pathway exhibit a greater susceptibility to herpes simplex encephalitis (HSE) compared to those with normal TLR-3 function (Guo et al., 2011; Herman et al., 2012; Lafaille et al., 2012; Reinert et al., 2012; Zhang et al., 2007). Mice treated with polyinosinic:polycytidylic acid (Poly:IC), a potent TLR-3 agonist, are also protected from HSE following intranasal challenge (Boivin et al., 2008). During the course of viral latency, virus-specific T cells appear to play a key role in maintaining the virus in the latent state. There is an inverse relationship between the number of $CD8^+$ T cells in the trigeminal ganglia and rate and extent of reactivation from latency as determined in a number of animal model systems (Hoshino et al., 2007). Virus-specific $CD8^+$ T cells surrounding latently infected ganglia form immunological synapses with polarization of pre-formed granules (Khanna et al., 2003). Treatment with anti-CD8 antibodies accelerates the rate of reactivation from trigeminal ganglia explants in murine models (St Leger and Hendricks, 2011) pin-pointing the importance of this immune cell population in controlling viral latency and reactivation. In this regard, hormonal imbalance and

stress are common triggers of HSV-1 reactivation and have been shown to reduce the function of $CD8^+$ T cells (Curtin et al., 2009; Freeman et al., 2008; Glaser and Glaser, 1998; Webster Marketon and Glaser, 2008). Taken together, these observations have been at the epicenter with respect to formulating mechanistic studies to examine the immunologic role of the $CD8^+$ T cells in regulating the pathway from viral latency to reactivation and recurrent disease.

There are numerous animals models which have been used to study infection, pathology, and immune responses of the eye, flank skin, and genital tract as previously reviewed (Kollias et al., 2014). The primary models used for studying immune responses to the HSV-1 have centered on the use of the mouse model that has involved the use of inbred, outbred, transgenic, mutated, and humanized mouse strains that vary in their degrees of susceptibility to HSV-1 infection. Mice of different genetic backgrounds, as well as genetically distinct HSV-1 isolates have been used to study numerous stages of HSV-1 infection, making results published by different investigators often difficult to interpret and/or compare. The ocular scarification model has been shown to be very effective at eliciting a strong immune response in the trigeminal ganglia that is likely involved in controlling the virus during the course of viral latency. However, there are far fewer studies performed in the mouse system to study infection and pathology of the virus in the lip, one of the most common forms of HSV-1 infection in the human population. A lip model was described in a series of papers in the 1980's (Kastrukoff et al., 1981), which has recently been used to determine the immune cell subsets required to restrict viral entry into the CNS of

C57BL/6 mice (Kastrukoff et al., 2010) and to map an NK-mediated locus of resistance that affects HSV-1 entry into the brain of adult mice (Kastrukoff et al., 2015) . This model compromises the epithelial barrier by abrading the outside of the lip, originally accomplished with a wooden spatula (Kastrukoff et al., 1982). The compromised barrier allows for a somewhat more direct access of the virus to the keratinocytes in the epithelium to establish infection. We propose that this model will continue to be another important tool in understanding HSV-1 replication and pathology in another anatomically important and commonly encountered tissue site where the majority of new HSV-1 infections are initiated. Consequently, we have defined the kinetics of HSV-1 replication and pathology in a susceptible strain of mice infected using the lip scarification model initially pioneered by Kastrukoff and colleagues now more than three decades ago to lay the foundation for the continued use of this model in studies to define the immune and therapeutic control of acute, latent, and recurrent infection and in studies to prevent, treat, and cure HSV infection.

2.3. MATERIALS AND METHODS

2.3.1. Virus, cells, and mice.

Plaque-purified isolates of HSV-1 strain McKrae (Hill et al., 1987) were grown in Vero cell monolayers in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were grown at 37°C in the presence of 5% CO_2. Wild-type C57BL/6 adult (procured at 12 weeks of age) male mice were purchased from Jackson Laboratories. All animal experiments

adhered to procedures and guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Drexel University. Mice were anesthetized with Avertin and the lower lip scarified with 10 vertical strokes of a 25-gauge needle covering an area approximately 5 mm^2. A viral inoculum of 6.0×10^5 plaque forming units (PFU) suspended in 10 µL of MEM was applied to the lower lip and allowed to adsorb for 1 hour. The mice were observed during the duration of anesthesia. Vero cell lysate, applied at a volume equal to the virus inoculum, was used as a vehicle control.

2.3.2. Titration of virus in tissue

At indicated time points mice were euthanized by CO_2 asphyxiation. The lower lip and TG were removed, weighed, and frozen in MEM containing 10% FBS. The tissue was stored at -80˚C until ready for processing. The frozen tissue was subjected to three freeze-thaw cycles, homogenized, and resuspended in 200 µL of serum-free MEM. The tissue homogenate was serially diluted in serum free MEM and then applied over a permissive Vero cell monolayer for viral quantitation studies. Virus was allowed to adsorb for 1 hour at 37°C and then Iscove's Modified Dulbecco's Medium (IMDM) and FBS (2%) in methylcellulose (1.5%) was added to the culture. The monolayer infection was allowed to proceed at 32˚C for 4 days; the plates were fixed and stained with crystal violet (1%). Individual plaques were counted and the final PFU was determined. The final PFU was normalized to the mass of the collected tissue for a final value of PFU per gram of tissue.

2.3.3. Macroscopy of lower lip

Mice were sacrificed at the indicated time points. The lower lips were visualized using a Leica EZ4HD stereo microscope at magnification factors of 0.8 and 3.0.

2.3.4. Histology

Mice were sacrificed at the indicated time points. The lower lips and TG were dissected and fixed in 4% formaldehyde for 24 hours. The tissues were transferred to 70% ethanol (EtOH), cleared, infiltrated, and embedded in paraffin. Sections were cut to a thickness of 4 µm and stained with hematoxylin and eosin (H&E) dyes (Malatesta, 2016) .Tissue processing and sectioning was performed by the pathology diagnostics laboratory at the Drexel University College of Medicine. Images were obtained with an Olympus IX81 microscope running CellSens Dimension version 1.13.

2.3.5. Immunohistochemistry of lip and TG sections

Tissue from mice sacrificed at the indicated time points were deparaffinized, cleared, and rehydrated. Heat-induced epitope retrieval was performed with sodium citrate buffer (10 mM, pH 6.0). Lip sections were treated with primary antibodies rat anti-mouse CD45 (30-F11-Tonbo) and rabbit anti-HSV-1 (Abcam) followed by overnight incubation at 4°C. Trigeminal ganglia sections were reacted with rat anti-mouse CD45 and rabbit anti-Neun (EPR12763-Abcam). T cells were detected in the lip using antibodies against CD3 (clone CD3-12 from Abcam), CD4 (clone 4SM95 from eBioscience) and CD8 (clone 4SM12 from eBioscience). T cells in the TG were detected using

monoclonal antibodies against CD4 (clone 15B12 from eBioscience) and CD8 (clone 4SM16 from eBioscience). Isotype matched antibodies which are non-specific for the antigens of interest were used for negative control experiments. Goat anti-rat Alexa 555 (Molecular Probes®) and Goat anti-rabbit Alexa 488 (Molecular Probes®) conjugated secondary antibodies were used for detection. Nuclei were counterstained with DAPI (Invitrogen). Sections were visualized using an Olympus IX81 fluorescent microscope running CellSense Dimension version 1.13.

2.3.6. Genome copy number

Mice were sacrificed at the indicated time points. The lower lip and TG were dissected and frozen in RNA*later*® RNA stabilization reagent (Thermo Fisher). The tissue was thawed and genomic DNA isolated using the GenElute™ Mammalian Genomic DNA Miniprep procedure as described by the manufacturer (Sigma Aldrich). Quantitative-PCR (qPCR) was performed using custom Taqman (Invitrogen) probes against the thymidine kinase gene (forward: GGCCCCCAACACGATGT, reverse: CGTGCTGGCGTTCGT) from the HSV-1 McKrae strain (accession: JX142173). Vero cells were infected with HSV-1 McKrae at an MOI of 0.01 and harvested after 48 hours. The PFU per mL of virus lysate was determined using a standard plaque assay and the genomic DNA of the virus lysate isolated as above. The virus lysate DNA was serially diluted and used to generate a standard curve for the qPCR reaction. The experimental sample cycle thresholds were interpolated and plotted on the standard curve

using GraphPad prism version 6.0. Genome copy numbers were made relative to the mass of isolated tissue to generate a genome per gram tissue value.

2.3.7. Poly:IC stimulation

High molecular weight Polyinosinic:polycytidylic acid (Poly:IC - Invivogen) was suspended in 10 mL of sterile water to a concentration of 1 mg/mL. Mice were stimulated with 100 µg of Poly:IC by IP injection or 10 µg of Poly:IC by direct injection into the lower lip 24 hours prior to infection. Sterile saline was used as vehicle control for Poly:IC. Mice were then infected with 6.0×10^5 pfus of HSV-1 McKrae. The mice were sacrificed 5 days post-infection and the lower lip harvested. The presence of cell free virus was assayed by plaque assay as described above.

2.3.8. Statistics

All statistics were done using GraphPad prism version 6.0.

2.4. RESULTS

2.4.1. Infected mice exhibit skin lesions similar to those in human infections

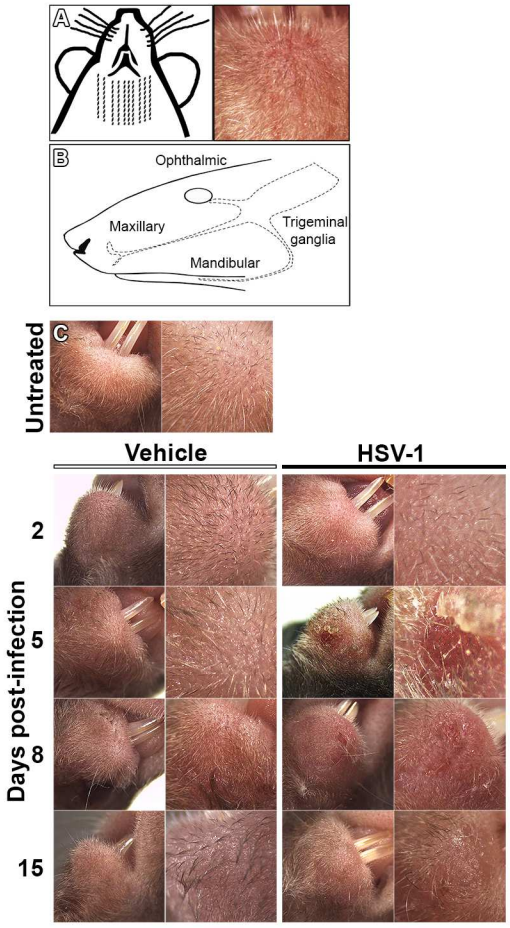
A majority of new HSV-1 infections in humans occur in the orofacial tissue, yet there are few available animal models for studying primary, latent, and reactivated infection in peripheral tissues and nerve tracts associated with the oral cavity and surrounding tissues. As one experimental approach to facilitate continued advancement of knowledge concerning this area of HSV-1-associated

pathogenesis and disease, the lip scarification model (Kastrukoff et al., 1981; Kollias et al., 2014) has used a needle to compromise the external barrier of the lip prior to experimental application of virus to the scarified area to consistently, and reproducibly initiate the infection. To this end, the lower lip was scratched using a 25-gauge needle (Fig. 2.1.A). Based on prior observations (Kastrukoff et al., 1988), this experimental protocol has been shown to deliver virus to cells in the epithelial and subepithelial regions of the lip. The virus gains access to sensory nerves with subsequent translocation of virus in the retrograde direction towards the trigeminal ganglia (TG) via the mandibular nerve branch (Fig. 2.1.B).

After inoculation with vehicle or virus, mice were sacrificed at the indicated time points and the lower lip was subjected to pathological examination. The untreated and vehicle exposed mice showed no signs of skin pathology (Fig. 2.1.C). Virus-infected animals exhibited erythematous vesicular lesions present on the lower lip on day 5 post-infection (second row). There were also initial signs of crusting over of the lesions. The lesions were still healing 8 days post-infection (third row) and had completely resolved by 15 days post-infection (last row). These results closely resemble the time course of human disease which has vesicular lesions in the first week that usually resolve during the second week.

Figure 2.1. Infected mice display clinical lesions. (A) Schematic of lip scarification with vertical scratches. Dashed lines indicate location of scratches (left). Image of mouse lip from the same location modeled in the cartoon (right). (B) Cartoon of the mouse trigeminal ganglia with sensory branches labeled. (C) Macroscopic image of the mouse lip untreated (top) or scratched and inoculated with either vehicle (left) or $6*10^5$ PFU of HSV-1 McKrae (right). At indicated time points, mice were sacrificed and the lower lips imaged using a dissection microscope. One experiment with n=3 mice.

Figure 2.1.


A
B
Ophthalmic
Maxillary
Trigeminal ganglia
Mandibular
C
Untreated
Vehicle
HSV-1
Days post-infection
2
5
8
15

2.4.2. Infected lips display histopathology associated with HSV-1 infection

In order to demonstrate that the skin lesions contained cytological pathology consistent with HSV-1 infection, samples were also processed for histological examination. The unexposed lower lip showed a thin epidermis that was 1-2 cells thick. Underlying the epidermis was the dermal layer containing dense collagen fibers, elongated fibroblasts, and resident leukocytes (Fig. 2.2.A). In the hypodermis layer were hair fibers, muscle fibers, sebaceous glands and adipocyte deposits. In mice exposed to the vehicle, a thickening of the epidermis (acanthosis) and infiltration of inflammatory cells were observed. Crust formation was also observed over the site of epidermal thickening and inflammatory cell infiltration. There was no extensive damage to the epidermal or dermal layers of the lip when exposed to the vehicle (Fig. 2.2.B, left). Mice infected with HSV-1 showed extensive damage in the epidermal and dermal layers. Multi-nucleated cell bodies containing epithelial cells with chromatin margination and cellular bodies lacking chromatin material (orthokeratosis) were observed in the animals exposed to virus (Fig. 2.2.B, right).

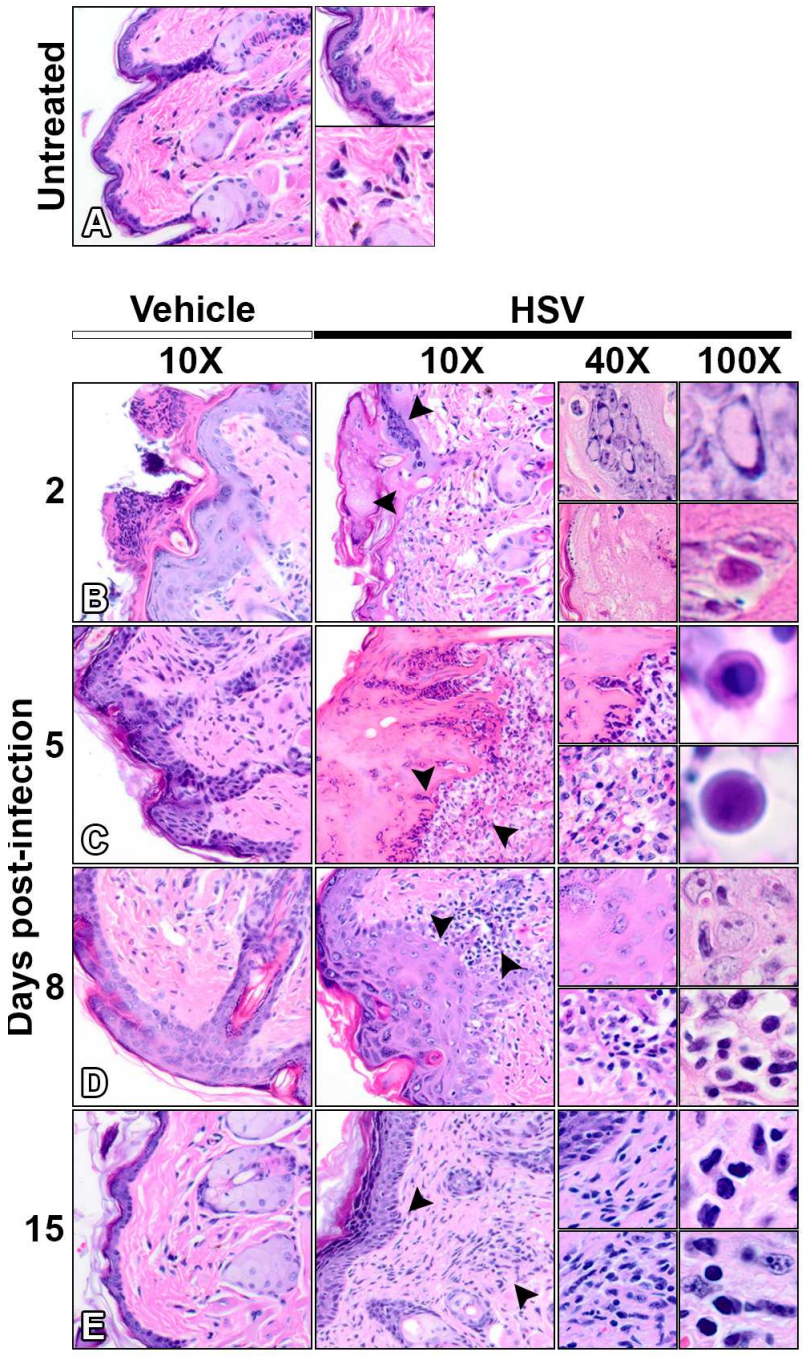
Mice exposed to vehicle alone for 5 days continued to show epidermal thickening and inflammation, but no additional damage to the dermis or epidermis (Fig. 2.2.C, left). However, mice exposed to virus preparations showed areas of complete epidermal destruction. The lesions were covered with a very thick layer of crust. The crust was composed of proteinaceous material, degenerated keratinocytes, and necro-inflammatory debris. Immediately below the crust, the dermis was severely disrupted with many dying and dead cells

containing Cowdry type A nuclear inclusion bodies (Fig. 2.2.C, right). By day 8 post-infection, mice infected with virus showed signs of recovery and healing. A smaller thickness of crust was observed over areas of healing. Areas of healing had a restored epidermal layer that was very thick indicating epidermal hyperplasia. However, cellular inflammation persisted beneath the thickened epidermal layers. Apoptotic bodies could be observed, but in lower numbers than observed at earlier time points (Fig. 2.2.D, right).

At 15 days post-infection, the epidermal layer still appeared thick. In contrast to the lips observed on day 8, the thickness was due to numerous cell layers and not hyperplasia of the epidermal cells. A strong inflammatory infiltrate remained in the lip (Fig. 2.2.E, right). These results indicate that during the active primary infection in the skin, initial replication occurs in the epidermal layer. As the infection proceeds, destruction of the epidermal layer and disruption of the dermis occurs and is covered by a thick crust. Immune cell infiltration was observed at the site of infection. Pathology is limited to the epidermal and dermal layers; no pathology could be observed in the hypodermis. By day 8, the epidermal layer was restored with the remaining pathology observed as a thick area of epithelial hyperplasia. By day 15 post-infection, the lesion consisted of a thickened epidermis with signs of inflammation.

Fig. 2.2. Histopathology of lips infected with HSV-1. Mice were scratched and inoculated with either vehicle (left) or 6.0×10^5 PFU HSV-1 McKrae (right). (A-E) at indicated time points, mice were sacrificed and the lips processed for histological sectioning and staining. Arrowheads point to regions of interest that are highlighted by high power insets at 40X or 100X magnification. One representative experiment with n=3 mice.

Figure 2.2.

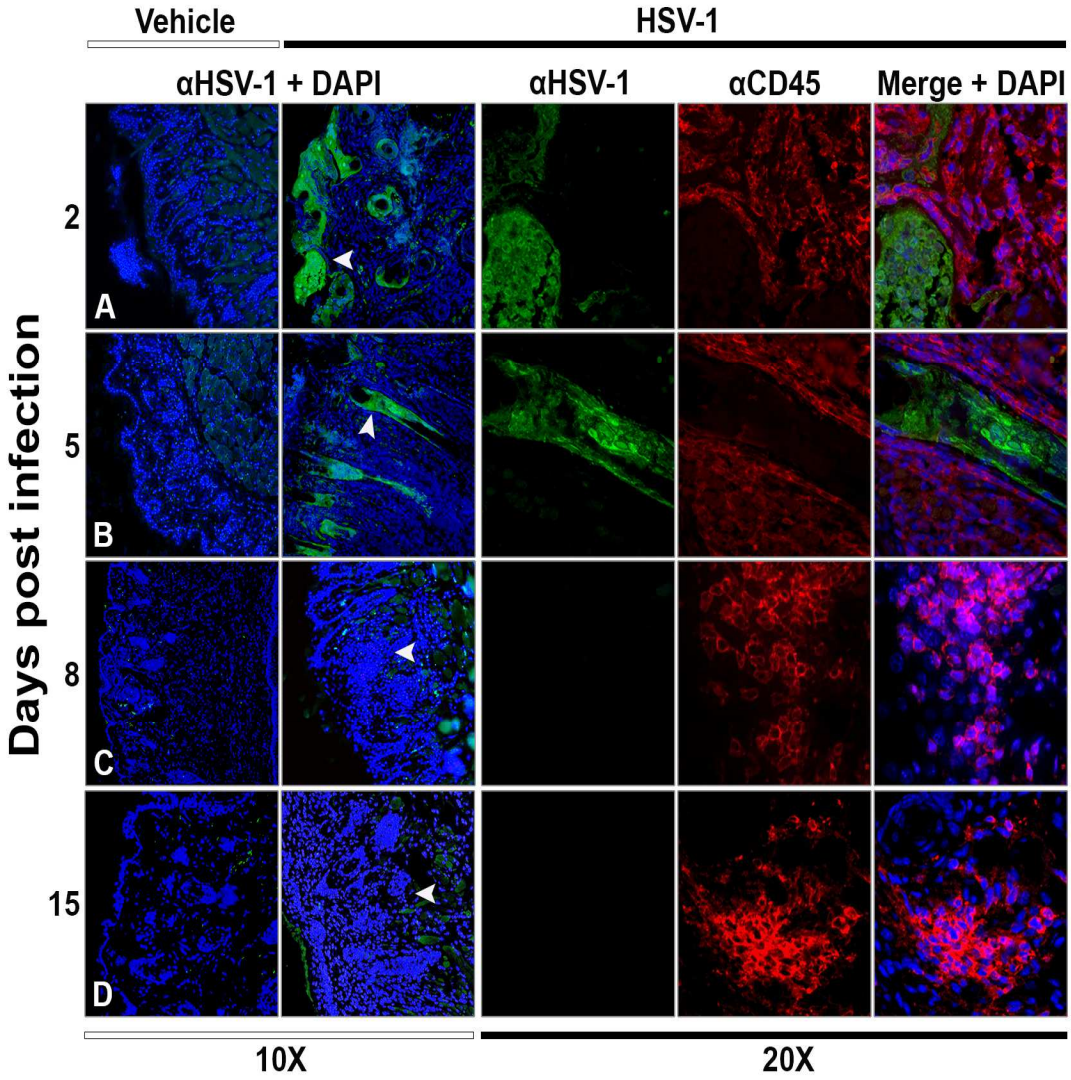

Untreated
A
Vehicle
HSV
10X
10X
40X
100X
Days post-infection
2
5
8
15
B
C
D
E

To correlate pathology in the lip with presence of virus replication, microscopic immunohistochemistry was used to detect viral antigen and demonstrate immune cell infiltration into the lip tissues infected with HSV-1. A polyclonal antibody preparation directed against HSV-1 (Abcam) was used in conjunction with an anti-CD45 monoclonal antibody (clone 30-F11, provided by Tonbo). CD45, a protein tyrosine phosphatase receptor that has been shown to be found on all hematopoietic cells, was used to identify leukocytes. The unexposed lip contained no viral antigen and few $CD45^+$ cells in the dermal layer of the lip (data not shown). In mice infected with HSV-1, large amounts of viral antigen were observed in the epidermal layer in mice at day 2 post-infection (Fig. 2.3.A). We were able to correlate viral antigen-positive cells with DNA staining pattern indicating chromatin margination. We also observed viral antigen in the hypodermal layer in hair follicles. At day 2 post-infection, $CD45^+$ cells were observed in close proximity to areas also containing HSV-1 antigen, as determination immunohistochemistry. At day 5, HSV-1 antigen was diminished and largely limited to the dermal layer below the crust. Dense areas of viral antigen were observed in hair follicles of the hypodermis. There was also a large accumulation of $CD45^+$ cells surrounding the HSV-1 antigen-positive hair follicles (Fig. 2.3.B). On day 8, no viral antigen was observed in the lip. However, large accumulations of $CD45^+$ cells were observed in areas of epithelial hyperplasia at this time point (Fig. 2.3.C). Finally, on day 15 post-infection, no viral antigen was present, but there were still numerous $CD45^+$ inflammatory cells present in the lip. (Fig. 2.3.D). These results demonstrated that the pathology present in the lip

was closely associated with the presence of viral antigen. Early in the infection, there was clear presence of viral antigen and detectable virus-induced pathology in the lip. However by day 8, no viral antigen was present in the lip and no pathology was observed in the lip. The remaining immune cells detected at day 15 represented the final stages of the healing process and the area that previously supported viral replication during acute primary infection.

Figure. 2.3. Infected mice display HSV-1 antigen in the lower lip. Mice were exposed to vehicle (left) or 6.0×10^5 PFU of HSV-1 McKrae (right). (A-D) Mice were sacrificed at indicated time points and processed for histological sectioning. Sections were reacted with antibodies against HSV-1 (green) and CD45 (red) and counterstained with DAPI (blue). Arrowheads point to regions of interest highlighted by high power panels. One experiment with n=3 mice.

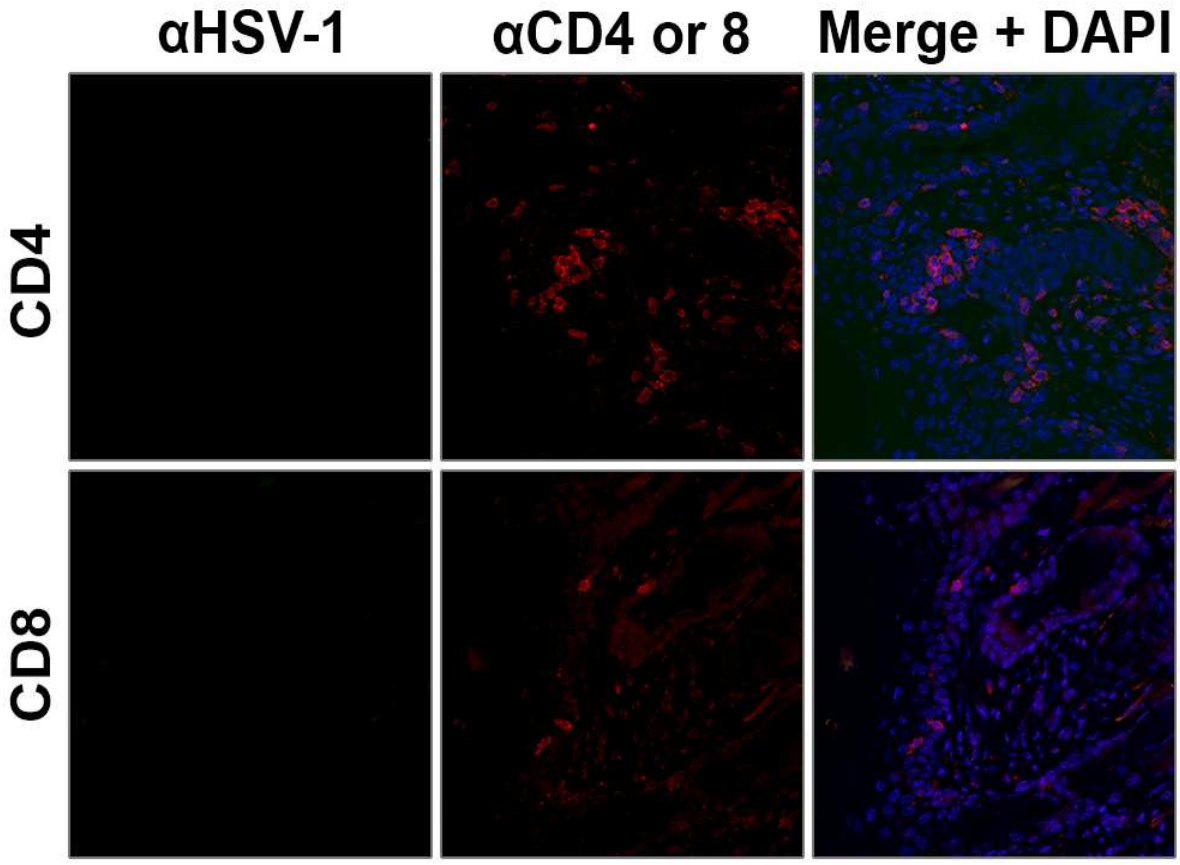
Figure 2.3.


Vehicle
HSV-1
αHSV-1 + DAPI
αHSV-1
αCD45
Merge + DAPI
Days post infection
2
5
8
15
A
B
C
D
10X
20X

The adaptive immune response plays an important role in resolving the primary infection in the periphery. $CD4^+$ T-helper cells coordinate immune responses to clear viral infections while $CD8^+$ cytotoxic T cells directly caused apoptosis of infected cells. Antibodies specific for T-lymphocyte markers were used to detect the presence of $CD4^+$ T cells and $CD8^+$ T cells in the lip. Preparations of polyclonal antibodies against HSV-1 and monoclonal antibodies against CD4 (clone 4SM95 provided by eBioscience) or CD8 (clone 4SM15 from eBioscience) were used on lip sections from mice harvested 15 days post-infection. This time point is after resolution of the primary infection in the lip (Fig. 2.7.) but it is anticipated that responding adaptive immune cells would still be present. Immunohistochemistry revealed a large population of $CD4^+$ T cells in the lip still present in the lip. $CD4^+$ T cells were found in close proximity to hair follicles and throughout the dermis layer (Fig. 2.4., top). There was a smaller population of $CD8^+$ T cells in the lip which appeared to be predominantly in the epidermal layer, but some were also near hair follicles (Fig. 2.4., bottom). There was no HSV-1 antigen straining present which confirmed that the peripheral infection had been resolved by 15 days post-infection.

Figure 2.4. $CD4^+$ and CD8 T cells were shown to be present in the lower lip. Mice were treated with 6.0×10^5 PFU HSV-1 McKrae and then sacrificed 15 days post infection. Tissue sections were treated with antibodies against HSV-1 (green) and CD4 or CD8 (red) and counterstained with DAPI (blue). One experiment with n=3 mice.

Figure 2.4.



Infection of innervating sensory neurons in the periphery leads to establishment of latency in the TG as shown previously (Hill et al., 1975). The TG is infiltrated with immune cells and eventually HSV-1-specific T cells are observed surrounding latently infected neurons. Consistent with these previous observations, sections of TG were prepared at increasing time points after the initial infection of the lip to determine if immune cells infiltrate the nerve following lip scarification and virus inoculation. The TG tissue derived from untreated and control animals exposed to vehicle alone showed a large amount of cellularity from satellite cells and Schwann cells (Fig. 2.5). However, beginning on day 5 post-infection, a large increase in the number of immune cells was observed in the nerve and those immune cells that were observed were associating with neuronal cell bodies (Fig. 2.5, arrowheads). Immunohistochemistry utilizing monoclonal antibodies directed against CD45 demonstrated that these cells were of hematopoietic origin and likely immune cells responding to viral infection of innervating sensory neurons. Minimal immune cell infiltration was observed on day 2 post-infection; possibly reflecting the time it takes for the virus to travel retrograde from the sensory neuron axon innervating the lip to the neuronal cell body in the TG and the time for the immune response to get deployed in the TG. The $CD45^+$ cells remained in the TG through day 15 post-infection, some of which are $CD3^+$ $CD4^+$ or $CD3^+$ $CD8^+$ T cells which will be retained in the TG during latency (Fig. 2.6).

Figure 2.5. CD45^{+} cells infiltrate the trigeminal ganglia of infected mice. At indicated time points, the trigeminal ganglia from mice exposed to vehicle or 6.0×10^{5} PFU HSV-1 McKrae were processed for histological sectioning. The trigeminal ganglia were stained with H&E (left) or treated with antibodies (right) against Neun (green) and CD45 (red) and counterstained with DAPI (blue). Arrowheads point to regions where cellular infiltration is evident. One representative experiment with n=3 mice.

Figure 2.5.

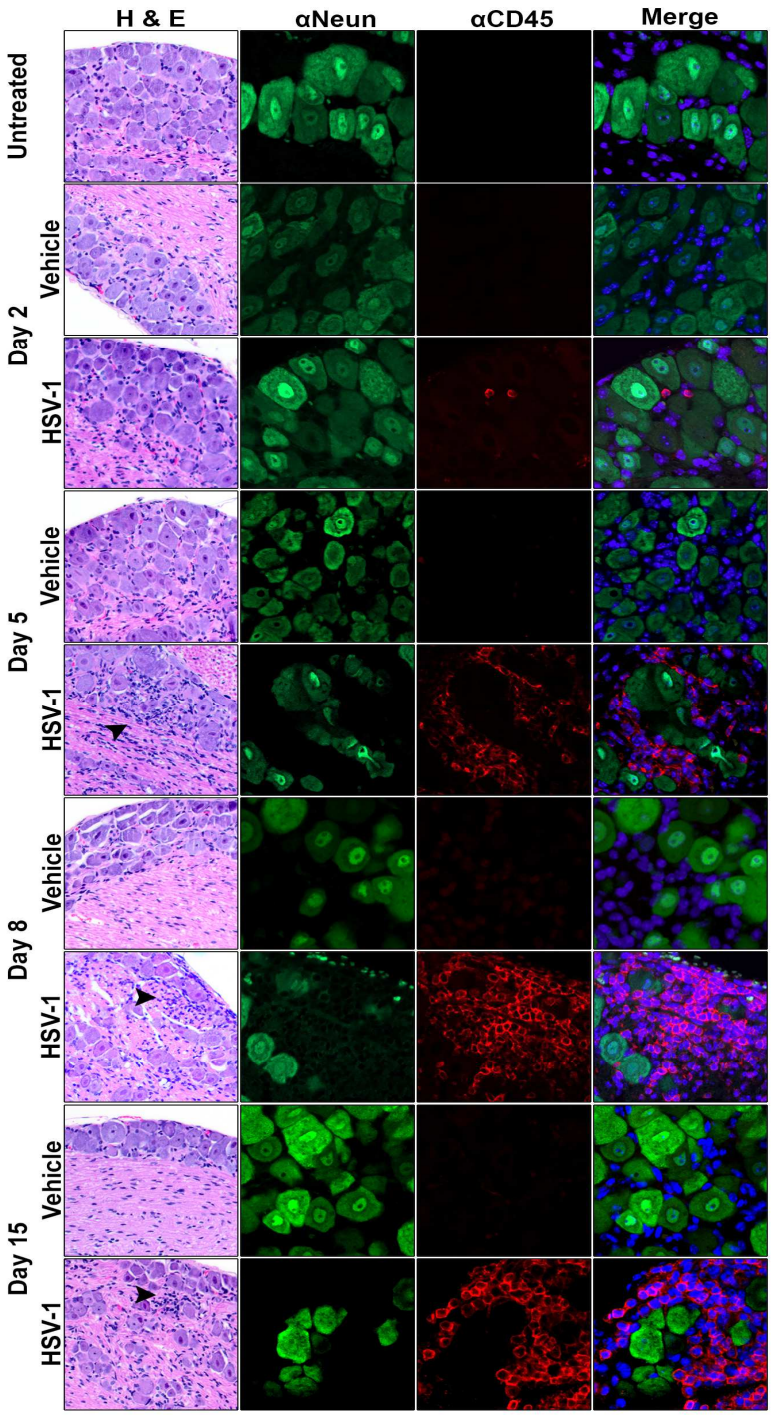
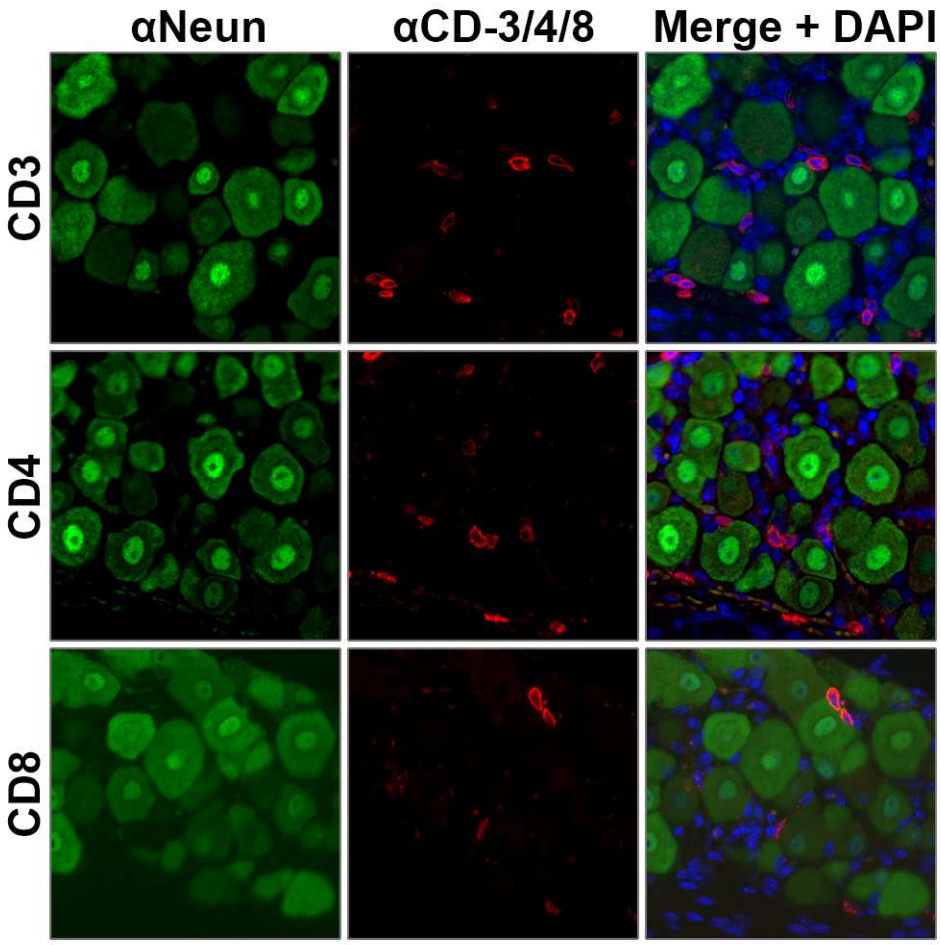

H & E
αNeun
αCD45
Merge
Untreated
Day 2
Vehicle
HSV-1
Day 5
Vehicle
HSV-1
Day 8
Vehicle
HSV-1
Day 15
Vehicle
HSV-1

Figure 2.6. $CD4^+$ and $CD8^+$ T cells infiltrate the trigeminal ganglia. Mice were infected with 6.0×10^5 PFU HSV-1 McKrae and then sacrificed 15 days post infection. Tissue sections were treated with antibodies against HSV-1 (green) and CD4 or CD8 (red) and counterstained with DAPI (blue). One representative experiment with n=3 mice.

Figure 2.6.



2.4.3. HSV-1 replicates in the lip before establishing latency in the trigeminal ganglia

To determine the time frame of viral replication in both the lip and TG, mice were sacrificed at indicated time points and tissue was subsequently collected from the lip and TG. Peak virus replication (1.90×10^6 pfu/gram) was observed in lip tissue day 2 post-infection. This level of virus production in the lip continued to day 5 post-infection (2.0×10^6 PFU/gram) followed by a decrease in replication to undetectable levels by day 8. Virus replication in the TG was not evident until day 5 post-infection (peaking at 2.0×10^4 PFU/gram), correlating with the observed increase in infiltrating immune cells at this time point (Fig. 2.4, row 5). At day 8, cell-free virus production was below the level of detection in the TG and remained so through the remainder of the time course (Fig. 2.7, left). While replicating virus decreased to undetectable levels, we hypothesized that there would still be viral genomes present in the TG. Genomic DNA was also isolated from the lips and TGs at the indicated times after infection to assay for viral genome copy numbers by quantitative PCR. High levels of HSV-1 genomes were present in the lip at days 2 and 5 post-infection, peaking at 1.5×10^5 genome copies per gram tissue. The number of viral genome copies decreased dramatically by day 8 but was still detectable at 8.0×10^3 copies per gram tissue. The number of viral genomes continued to decrease at day 15 but was still detectable at 6.0×10^3 copies per gram tissue in the lip (Fig 2.7, right). This was an unexpected result as cell-associated or cell-free infectious virus was no longer detectable after 5 days post-infection by plaque assay. The nature of the HSV-1

genomes detectable in the lip at later times post-infection will be future investigation in Chapter III. Retention of the HSV-1 genome at peripheral sites in the absence of detectable viral infectivity in cell-free homogenates has been observed previously in ocular tissues and other peripheral sites (Higaki et al., 2015).

With respect to the TG, HSV-1 genome copies in the TG were detectable by day 2 post-infection. The number of detectable viral genome copies was shown to increase at day 5 and established an apparent base line around 1.0×10^5 genome copies per gram tissue. The level of HSV-1 genome copy number retained in the TG was consistent at days 5, 8, and 15 post-infection (Fig. 2.7, right).

Finally we tested the model by stimulating mice with the immunogenic molecule poly:IC. It has been established that patient's harboring mutations in the TLR3 pathway have an increased susceptibility to herpes simplex encephalitis. Poly:IC has been used in animal models of herpes simplex encephalitis to protect animals from lethal challenge. Poly:IC is a synthetic double stranded RNA molecule which is a potent stimulator of the TLR3 pathway, which plays a role in anti-viral responses in the immune response. Activation of the TLR3 pathway leads to induction of the antiviral state in uninfected cells and secretion of interferons which would initiate the antiviral response of the innate immune system. Given this, poly:IC was used prior to infection to determine if treatment would aid in clearance of infectious virus.

Mice were treated with 100 µg of poly:IC suspended in water 24 hours prior to infection. Mice were sacrificed 5 days post-infection and the tissue analyzed for infectious cell free virus. Mice which were treated via intraperitoneal injection had a mean viral load of 1.4×10^6 pfus per gram of tissue compared to 4.0×10^5 pfus per gram tissue in the saline treated animals. This indicates that there was no significant reduction in virus replication in the lip. It is possible that systemic administration of the poly:IC did not appreciably activate antiviral immune responses in the lower lip where the virus was administered and replication occurred. We treated mice with 10 µg of poly:IC injected directly into the lower lip prior to viral infection. Mice were harvested 5 days post-infection and the tissue valuated for infectious cell free virus. The mean viral load in the lips of these mice was 1.3×10^6 pfus per gram tissue compared to 6.2×10^5 pfus per gram tissue in saline treated animals.

Figure 2.7. Kinetics of HSV-1 replication in the lip and trigeminal ganglia of infected mice. Mice were infected with 6×10^5 PFU HSV-1 McKrae. At indicated time points, the lip and trigeminal ganglia were isolated and analyzed for infectious virus by plaque assay (left) and viral genome copies by qPCR (right). The dotted line indicates limit of detection by plaque assay. PFU and genome copy values were made relative to the mass of the tissue analyzed. Closed squares indicate results obtained with lip tissue. Open circles represent the results obtained in trigeminal ganglia tissue. Data are from 1 experiment with n=5 mice per time point.

Figure 2.7.

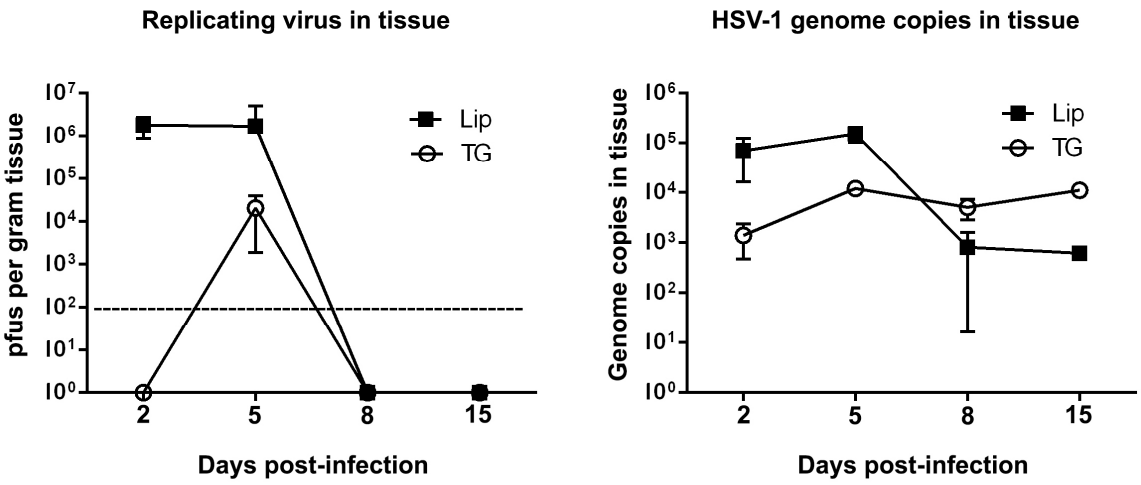
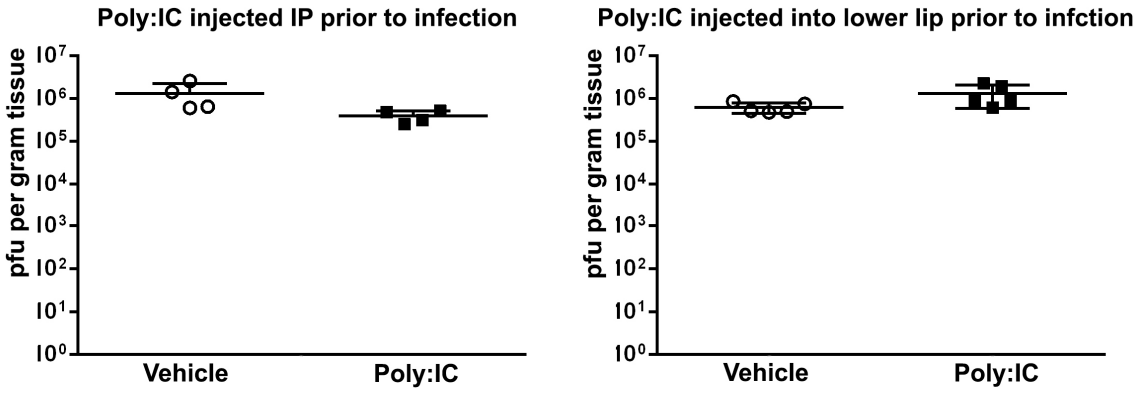

Replicating virus in tissue
pfus per gram tissue
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Lip
TG
2
5
8
15
Days post-infection
HSV-1 genome copies in tissue
Genome copies in tissue
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Lip
TG
2
5
8
15
Days post-infection

Figure 2.8. Treating mice with poly:IC prior to infection does not reduce virus replication in the lip. Mice were treated with poly:IC 24 hours prior to infection. Mice were treated with intraperitoneal (IP) injection of 100 ug (left) or direct lower lip injection of 10 ug (right) of poly:IC. The mice were sacrificed and the lower lips harvested 5 days post-infection. These data are from 1 experiment n=4 mice per group (left) or 5 mice per group (right).

Figure 2.8.


Poly:IC injected IP prior to infection
pfu per gram tissue
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Vehicle
Poly:IC
Poly:IC injected into lower lip prior to infction
pfu per gram tissue
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Vehicle
Poly:IC

2.5. DISCUSSION

The first reported usage of the lip model was in 1981 (Kastrukoff et al., 1981) and was later modified to some extent by initiating HSV-1 infection by abrasion of the mouse lip with a wooden spatula (Kastrukoff et al., 1982). The lip infection model has been used successfully to study HSV-1 entry into the CNS, HSV-1-induced demyelination (Kastrukoff et al., 1988), and genetic resistance to HSV-1 encephalitis in different strains of laboratory animals (Kastrukoff et al., 2012). These studies demonstrated that using this model, mice could be successfully infected with HSV-1 and that virus could gain access to the CNS as well as the TG. These studies did not seek to define the kinetics of viral replication or the corresponding immune responses in the lip or peripheral nervous system. However, to date, the model has not been widely adopted and instead, most studies involving HSV-1 pathogenesis have used the flank, foot-pad, or ocular scarification model for studying virus replication and pathogenesis as well as the associated immune responses. Because most new human HSV-1 infections occur in the oro-facial mucosa and surrounding epithelial tissues, we propose that the lip inoculation model would be very physiologically relevant for studying human disease caused by HSV-1. In order to validate the model for studying human disease, we first sought to define the viral replication kinetics, host pathology, and immune response to viral HSV-1 infection.

It was clear that the scarification of the lip did not cause severe or lasting damage to the lip both macroscopically and microscopically (Fig. 2.1 and 2.2). Erythematous lesions were observed in the lower lip in virus-infected animals

that were very similar to what has been observed previously in humans with primary infection (Arduino and Porter, 2008). Histopathological examination (Fig. 2.2) and immunohistochemistry (Fig. 2.3) demonstrated the presence of HSV-1 antigen in the lip with related pathology in the epidermis. A strong inflammatory infiltrate was observed at all points during the acute infection. It was very evident from the accumulated HSV-1 antigen and $CD45^{+}$ staining during the course of infection that the hair follicles were a prominent site of HSV-1 infection in the lip. Hair follicles have been shown to be innervated to induce secretion of sebum and contraction of erector pili. The presence of HSV-1 in the innervated hair follicles could represent a route of infection involving the peripheral nervous system outside of the sensory axons leading to the trigeminal ganglia. The kinetics of viral infection, replication, and pathogenesis were very similar to what has been observed in cases of human infection where most of the pathology is observed in the first week, with the lesions subsequently healing and resolving during the second week. In general, the viral replication and pathogenic kinetics shown in the mouse lip infection model mirror the results previously reported with respect to clinical signs and symptoms of disease observed in humans. This model could also be used for examining the pathology associated with HSV-1 infection of the oral tissue. Furthermore, this model would be extremely useful for studying new anti-viral compounds and their impact on reduction of viral replication, pathology, and inflammatory responses prior to clinical trials in humans.

As one of the key features to our experimental approach, C57BL/6 mice were selected as the target mouse strain because these animals have been shown to be one of the most resistant strains of mice with respect to viral entry into the CNS that often leads to a fatal encephalitic disease. Furthermore, adult mice were selected, to ensure a mature and competent immune system that facilitates the survival of primary disease and the establishment of a latent infection. The HSV-1 McKrae strain was selected because it represents a viral strain that has previously been shown to be neuroinvasive. The combination of a highly immunocompetent adult mouse with a genetic background including a high resistance to HSV-1 CNS viral penetration infected with the neuroinvasive McKrae strain of HSV-1 previously shown to be able to penetrate the peripheral nervous system has led to efficient entry of virus into the mouse TG subsequent to a mouse lip infection with the subsequent establishment of latency within the context of a robust immune system facilitating survival in the absence of CNS disease. Replicating virus was readily observed in the lip and TG that resolved by 8 days after initial infection. A similar pattern of viral replication was observed in the lip utilizing a qPCR assay designed for the detection of viral copies of the TK gene. However, instead of complete clearance of detectable viral genomes as determined by the quantitation of the TK gene in the lip, detectable genome copies remained at 8 and 15 days post-infection. This observation was somewhat unexpected since we theorized that along with resolution of pathology and replicating virus, intact viral nucleic acid would no longer be detected in lip tissues after the disappearance of viral infectivity. This result has suggested the

possibility of either low level long-term viral genome persistence in the lip in the absence of cell-free viral infectivity or non-neuronal latency in the lip. There have been numerous studies exploring the possibility that HSV-1 persists in a “latent state” within corneal tissues. Corneal transplants are a common procedure and have restored vision to numerous individuals. However there is evidence of transmission of HSV-1 from the corneas of infected individuals to HSV-1-negative transplant recipients (Higaki et al., 2015; Kaye et al., 1991). Corneal epithelial cells have been shown to be capable of supporting *in vitro* latency (Cook and Brown, 1986).

The lip model has also been shown to be useful for studying selected aspects of the immune response to viral infection as well as immunologic events post viral clearance. We detected $CD4^+$ and $CD8^+$ T cells in the lip 15 days post-infection (Fig. 2.4). Some of these cells will likely die during the immune cell contraction phase, but others will remain long lived in the lip where the virus was initially encountered. Lip tissue sections at 30 days post-infection have confirmed the retention of these cells long after the resolution of primary infection. Monoclonal antibodies which can detect CD4 and CD8 in formalin fixed paraffin embedded mouse tissue have only recently become available. It is believed that formaldehyde fixation damages the CD4 and CD8 antigens to a point that previously available antibody formulations were unable to detect them in paraffin embedded tissue. Extensive work has been performed to evaluate epitope retrieval techniques to make the antigen available, but to date the results have been unsatisfactory (Kim et al., 2004a, 2004b). There is compelling evidence that

virus-specific $CD8^+$ T cells play an important role in maintaining the virus in a noninfectious latent state. In addition, the virus-specific $CD8^+$ T cells in the TG belong to a recently described class of tissue resident memory T cells (T_{RM}) (Himmelein et al., 2011). The virus travels by retrograde flow from a peripheral site during primary infection to the TG where it establishes latency. We have observed $CD3^+$, $CD4^+$, and $CD8^+$ T cells in the TG 15 days post-infection (Fig. 2.6). These cells were dispersed in the TG and commonly found adjacent to neurons. We have also shown that the available clones for detecting CD4 (4SM9 from eBioscience) and CD8 (4SM15 from eBioscience) were producing non-specific staining of neuronal cell populations in the TG. Additional clones provided by eBioscience were able to detect the CD4 and CD8 antigens in the TG without the non-specific staining. The presence of these cells confirms the usefulness of the lip model for studying T cells responses to HSV-1 infection in the TG. We have been able to isolate and perform flow cytometry on immune cells from the TG following lip scarification and infection (data not shown). The role that immune cells play in maintaining the virus in a latent state is important for understanding the triggers of reactivation. Many common triggers including hormone imbalance and stress have inhibitory effects on $CD8^+$ T-cell function (Freeman et al., 2008; Sinani et al., 2013). Drugs that alter the immune system for treatment of other systemic conditions like rheumatoid arthritis (RA), chronic ulcerative colitis, and chronic plaque psoriasis could have negative consequences on the immune system's ability to control acute and recurrent infections (Bradford et al., 2009). The host pathogen interactions resulting from

treatment with drugs which specifically target immune cell responses can be explored using this model of infection.

For decades, investigators have been searching for a potential prophylactic or therapeutic vaccine for HSV-1. Recent work has shown that gD null (Wang et al., 2016) and gK null (Iyer et al., 2013) mutants might provide strong immunity against lethal HSV challenges in animal models. It would be useful to determine whether these potential vaccine candidates work in a physiologically relevant animal model system where a low to moderate dose of virus would be applied permitting the animal to recover from the acute infection. The efficacy of these vaccines to prevent or reduce the establishment of latency and recurrent infection could be studied with the HSV-1 lip scarification mouse model.

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Chapter III

Latency and reactivation from the lip and trigeminal ganglia in mice infected using the lip scarification model

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Kevin Egan conceptualized experiments, performed experiments, collected and analyzed data and wrote the chapter. Alexander Allen collected data. Drs. Stephen Jennings and Brian Wigdahl participated in the intellectual development, conceptualized experiments and critically evaluated all aspects of the chapter. Dr. Stephen Jennings is supported by developmental funds provided by the Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine

3.1. ABSTRACT

Lip scarification has been successfully used to model the acute events of HSV-1 infection in oral mucosa as well as the surrounding epithelial tissues. Here the lip scarification is used to model HSV-1 establishment of latency and reactivation in the laboratory mouse. Adult mice were inoculated with HSV-1 after scarification and sacrificed 30 days later. No infectious virus could be found in the lip and trigeminal ganglia after 30 days. However, HSV-1 genomes were detectable in the TG and expression of the major LAT transcript was detected. CD4+ and CD8+ T cells were detected in the area of the lip where mice were inoculated as well as in the trigeminal ganglia. Latent HSV-1 genomes in the trigeminal ganglia reactivated from latency when single cell TG suspensions were cocultured with indicator cells. Interestingly, the lip tissue was also capable of reactivating from latency when cocultured with indicator cells. These novel results could be indicative of peripheral latency with virus capable of reactivation from a peripheral site in standard co-cultivation assays under conditions where infectious was not detectable in cell-free homogenates.

3.2. Introduction

Herpes simplex virus type 1 (HSV-1) is a ubiquitous human pathogen that is endemic worldwide (Xu et al., 2006). The herpesviruses are unique in their ability to establish latent infections which persist for the lifetime of the host. During primary infection in the periphery, the virus infects sensory neurons innervating the peripheral tissue. The virus travels in a retrograde direction to replicate in cells of the trigeminal ganglia (TG). Following activation of the adaptive immune system, the replicating virus is cleared from the periphery and TG. With clearance of the acute infection, no replicating virus can be found in the peripheral tissue or TG, and virus is not actively being shed from infected individuals (Schulte et al., 2014). However, with exposure to certain triggers, the virus can reactivate from latency and replicate within the TG, traveling back down the axons to the periphery to replicate in epithelial cells and cause recurrent disease.

The latent virus is maintained in the nucleus of infected neurons. The viral genome persists as an episome which is bound up with nucleosomes bearing heterochromatin markers (Efstathiou et al., 1986a). During latency, no abundant viral gene expression is detected with the exception of the latency-associated transcript (LAT). The LAT is an 8.3 kb species which is processed to produce a 2 kb major LAT and 6.3 kb minor LAT species (Javier et al., 1988; Wagner et al., 1988). The minor LAT species is further processed to make microRNAs (miRNA) which bind to and reduce expression of viral lytic genes (Jurak et al., 2012; Pan et al., 2014; Schlee et al., 2009; Umbach et al., 2008). The major LAT transcript

functions to promote survival of the latently infected cells which are surrounded by virus-specific $CD8^+$ T cells in the trigeminal ganglia (Branco and Fraser, 2005). LAT appears to also be necessary for efficient reactivation from latency, though the mechanisms through which it accomplishes these functions are currently unknown.

There are numerous in vitro and in vivo models used to study latency in reactivation. In vitro systems range from quiescently infected fibroblasts to infection of neuronal cell lines (Scheck et al., 1989; Wigdahl et al., 1984, 1983, 1982a, 1982b, 1981). However, most in vitro systems require an unnatural repression of virus replication with either anti-viral drugs or mutant viruses lacking lytic genes to induce a latent state (reviewed in (Scheck et al., 1986; Wigdahl et al., 1982a) . Rabbit animal models of reactivation are popular because iontophoresis treatment of the eye reproducibly initiates reactivation. Reactivated virus can also be readily detected from tear swabs and do not require sacrifice and harvest of infected tissue (Cook et al., 1987). Unlike rabbits, the laboratory mouse does not undergo spontaneous reactivation (Feldman et al., 2002). However in vivo reactivation has been achieved using stressors such as hyperthermia, restraint stress, and hormonal injections as previously reviewed (Kollias et al., 2014)). The mouse has also led to numerous ex vivo model systems which involve plating explanted TG in culture. The explanted cultures reactivate from the stress of being surgically removed from the animal. The timing of reactivation can be altered with addition of nerve growth factor (NFG) (Wilcox et al., 1990; Wilcox and Johnson, 1987) or anti-CD8 antibodies (Khanna

et al., 2003). This has led to insights into how the function of CD8+ T cells in the TG and neuronal health affect the regulation of reactivation.

The mouse latency models principally focus on the eye and flank as sites of infection. However, most new human infections above the neck occur in the orofacial tissue. We recently characterized the replication kinetics and pathology of HSV-1 infection in the lip scarification model. This model uses a topical application of HSV-1 to the scratched lip of anesthetized mice (Kastrukoff et al., 1986). The virus replicates in the lip, and clinical lesions consistent with human infections have been observed. The virus gains access to sensory nerves and subsequently the TG. The immune response infiltrates infected tissues and clears replicating virus from the lip and TG. However, viral genomes can still be detected in the TG after resolution of the primary infection. Here we evaluate the lip scarification model for studying HSV-1 latency and reactivation in a mouse model.

Scarified mice were infected and then harvested 30 days post-infection. We detected latent genomes in the TG and related LAT expression. Explanted TGs were capable of reactivating from latency in culture, and infectious HSV-1 can be recovered by explanted cocultivation. Interestingly, explanted lips were also capable of reactivating from latency utilizing similar procedures. These surprising results shed new light on the nature of HSV-1 latency and reactivation in the periphery.

3.3. MATERIALS AND METHODS

3.3.1. Virus, cells, and mice

Plaque-purified isolates of HSV-1 strain McKrae (Hill et al., 1987) were grown in Vero cell monolayers in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). Cells were grown at 37°C in the presence of 5% CO_2. Wild-type C57BL/6 adult (procured at 12 weeks of age) male mice were purchased from Jackson Laboratories. All animal experiments adhered to procedures and guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Drexel University. Mice were anesthetized with Avertin and the lower lip scarified with 10 vertical strokes of a 25-gauge needle covering an area approximately 5 mm^2. A viral inoculum of 6.0×10^5 PFU suspended in 10 µL of DMEM was applied to the lower lip and allowed to adsorb for 1 hour. The mice were observed for the duration of anesthesia. Vero cell lysate, applied at a volume equal to the virus inoculum, was used as a vehicle control.

3.3.2. Titration of virus in tissue

At indicated time points, mice were euthanized by CO_2 asphyxiation. The lower lip and TG were removed, weighed, and frozen in DMEM containing 10% FBS. The tissue was stored at -80°C until ready for processing. The frozen tissue was subjected to three freeze-thaw cycles, homogenized, and resuspended in 200 µL of serum-free DMEM. The tissue homogenate was serially diluted in serum-free MEM and then applied to permissive Vero cell monolayers for viral quantitation studies. Virus was allowed to adsorb for 1 hour at 37°C and then

Iscove's Modified Dulbecco's Medium (IMDM) and FBS (2%) in methylcellulose (1.5%) was added to the culture. The infection of the monolayer was allowed to proceed at 37°C for 4 days; the plates were fixed and stained with crystal violet (1%). Individual plaques were counted and the PFU was determined. The PFU was normalized to the mass of the collected tissue for a final value of PFU per gram of tissue.

3.3.3. Histology

Mice were sacrificed 30 days post infection and the lip and TG were surgically removed. The tissue was fixed in 4% formaldehyde for 24 hours, transferred to 70% ethanol, cleared, infiltrated, and embedded in paraffin. Sections were cut to a thickness of 4 μm and stained with hematoxylin and eosin (H&E) dyes (Malatesta, 2016). Tissue processing and sectioning was performed by the pathology diagnostics laboratory at Drexel University. Images were obtained with an Olympus IX81 microscope running CellSens Dimension version 1.13.

3.3.4. Immunohistochemistry of lip and TG tissue sections

Processed and sectioned tissue from sacrificed mice were deparaffinized, cleared, and rehydrated. Heat-induced epitope retrieval was performed with sodium citrate buffer (10 mM, pH 6.0). T cells were detected in the lip using antibodies against CD3 (clone CD3-12 from Abcam), CD4 (clone 4SM95 from eBioscience), and CD8 (clone 4SM12 from eBioscience) combined with a polyclonal antibody against HSV-1 (Abcam). T cells in the TG were detected using monoclonal antibodies against CD3 (clone 3D3-12 from Abcam), CD4

(clone 15B12 from eBioscience), and CD8 (clone 4SM16 from eBioscience). Neuronal cell bodies were detected using a monoclonal antibody specific for Neun (clone EPR12763-Abcam). All the primary antibodies were used at a concentration of 5 µg and incubated overnight at 4˚C. Goat anti-rat Alexa 555 (Molecular Probes®) and goat anti-rabbit Alexa 488 (Molecular Probes®) conjugated secondary antibodies were used for detection. Secondary antibodies were used at a concentration of 2 µg and incubated 1 hour at room temperature. Nuclei were counterstained with DAPI (Invitrogen). Sections were visualized using an Olympus IX81 fluorescent microscope running CellSense Dimension version 1.13.

3.3.5. Genome copy number

Mice were sacrificed at the indicated time points. The lower lip and TG were dissected and stored in RNA*later*® RNA stabilization reagent (Thermo Fisher). The tissue was minced and genomic DNA isolated using the GenElute™ Mammalian Genomic DNA Miniprep procedure as described by the manufacturer (Sigma Aldrich). Quantitative-PCR (qPCR) was performed using custom Taqman (Invitrogen) probes against the thymidine kinase gene (forward: GGCCCCCAACACGATGT, reverse: CGTGCTGGCGTTCGT) from the HSV-1 McKrae strain (accession: JX142173). Vero cells were infected with HSV-1 McKrae at an MOI of 0.01 and harvested after 48 hours. The PFU per mL of virus lysate was determined using a standard plaque assay and the genomic DNA of the virus lysate isolated as above. The virus lysate DNA was serially diluted and used to generate a standard curve for the qPCR reaction. The experimental

sample cycle thresholds were interpolated and plotted on the standard curve using GraphPad prism version 6.0. Genome copy numbers were made relative to the mass of isolated tissue to generate a genome per gram tissue value.

3.3.6. LAT expression

Mice were sacrificed at indicated time points. The lower lip and TG were dissected and stored in RNA*later*® RNA stabilization reagent (Thermo Fisher). RNA was isolated from tissue using PureLink™ RNA Mini procedure (Invitrogen) as previously described by the manufacturer. Complementary DNA (cDNA) was generated using the High-Capacity cDNA Reverse transcription procedure (Thermo Scientific) as previously described by the manufacturer. PCR was performed using primers against the major LAT species (forward: GGCGTCGGCGACATCC, reverse: CCCGAGTGTTCATCTCAGGC) (Nicoll et al., 2016) from the HSV-1 McKrae strain (accession: JX142173).

3.3.7. Tissue digestion

Mice were sacrificed 30 days after infection and tissue harvested. The lip and TG were immediately placed in DMEM on ice. The lip was digested using a cocktail of 3 mg/mL collagenase type 4 and 0.15 mg/mL DNAse 1 (Worthington Biochemical) at 37°C in the presence of 5% CO_2. The digestive enzymes were quenched with FBS-containing media and the tissue pelleted. The tissue was resuspended in 1 mL of growth media. Remaining large pieces of tissue were dispersed using the plunger of a needle. Cells were pelleted and resuspended in 1 mL of growth media.

The TG was digested using a Liberase TM (Roche Diagnostics) solution at a concentration of 2 units/mL for 1 hour in a 37°C water bath. The tissue was dispersed using a p200 pipette tip. The tissue was pelleted and resuspended in 1 mL of growth media.

3.3.8. Coculture of digested tissue and media sampling

The resuspended digested tissue was plated evenly across Vero cells which were grown to 50% confluency. The digested tissue solution was allowed to settle on the Vero cells for 1 hour at 37°C in the presence of 5% CO_2. FBS containing media was then added to the cultures and incubated for 10 days at 37°C in the presence of 5% CO_2. The media from the cocultures was sampled every day and stored at -80°C. Fresh complete growth media was replaced in the cultures after media sampling. At the end of the 10 days, the coculture monolayers were visualized and brightfield images were taken using an Olympus IX81 fluorescent microscope running CellSense Dimension version 1.13. The plates were then stained using Hematoxylin & Eosin dyes for plaque morphology staining. The collected media was used in standard plaque assay to determine when infectious HSV-1 virions could be detected in the media.

3.3.9. Immunocytochemistry of isolated media samples

Vero cells were grown to a confluency of 80% on autoclaved glass coverslips. Collected media from the reactivated tissue was plated over Vero cells and incubated at 37°C in the presence of 5% CO_2. The cells were fixed 48 hours later with 1% formaldehyde at 4°C and permeabilized with 0.05% Triton-X-100. Polyclonal antibodies specific for HSV-1 were incubated overnight at 4°C to

detect HSV-1 antigen in the cultures. Goat anti-rabbit Alexa 555 (Molecular Probes®) conjugated secondary antibodies were used for detection of the primary antibodies. Cells were counterstained with DAPI (Invitrogen). Cells were visualized using an Olympus IX81 fluorescent microscope running CellSense Dimension version 1.13.

3.3.10. Plaque morphology staining

Vero cells were grown on autoclaved glass coverslips to a confluency of 80%. Media sampled from cultured coculture wells was plated on Vero cells and incubated at 37°C in the presence of 5% CO_2. The cells were then covered with Iscove's Modified Dulbecco's Medium (IMDM) + FBS (2%) in Methylcellulose (1.5%) and cultured for 4 days. When plaques were to be observed microscopically, the cells were fixed for 1 hour using 4% formaldehyde (10% neutral buffered formalin). The methylcellulose was removed and the cells washed with PBS + 0.05% Tween-20. The cells were permeabilized using 0.05% Triton-X-100 for 10 minutes and washed again. The nuclei were stained using Gills Hematoxylin III and the cell cytoplasm stained using Eosin Y. After washing and dehydrating, the cells were mounted on glass slides and images were obtained with an Olympus IX81 microscope running CellSens Dimension version 1.13. Plaques from collected media were compared to plaques from stock HSV-1 strains McKrae, 17+, and RE.

3.3.11. Statistics

All statistics were done using GraphPad prism version 6.0.

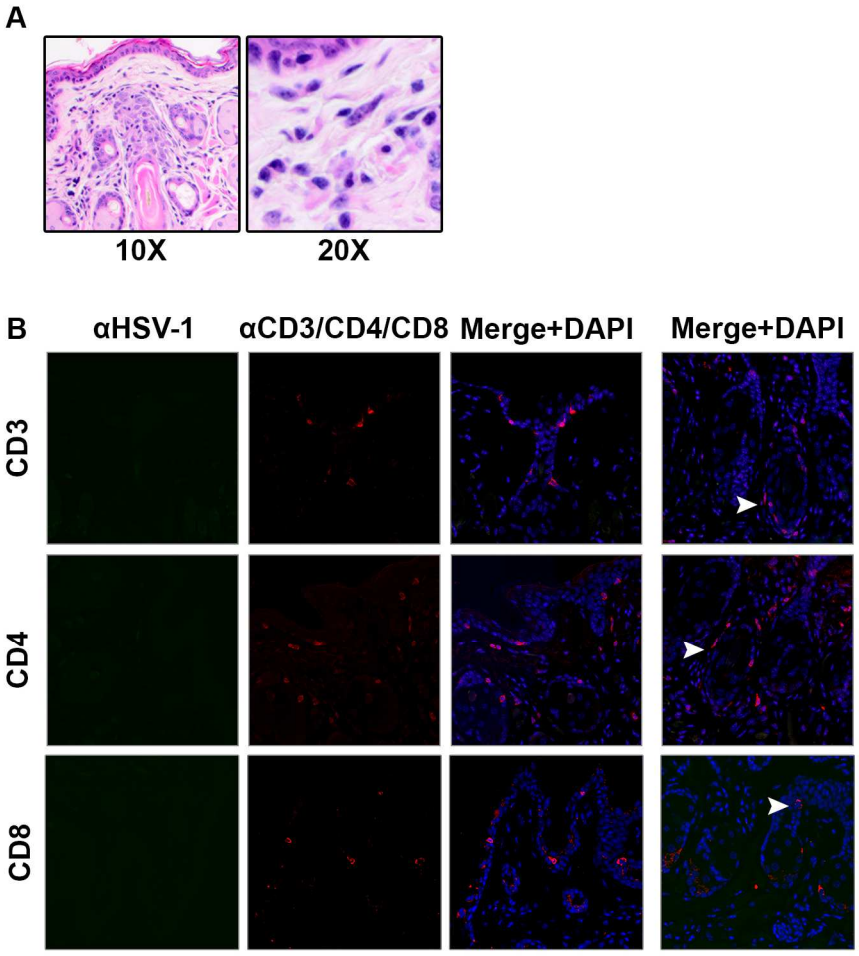
3.4. RESULTS

3.4.1. Primary infection has resolved 30 days post-infection

To determine if the lip scarification model can be used for studying HSV-1 latency and reactivation, mice were infected and incubated for 30 days before analyzing them for presence of latent virus. After 30 days, the primary infection will have resolved, and the virus has time to establish latency in the TG. We observed no pathology in the lip by histology in mice 30 days post-infection. The upper lips from infected mice contained a normal epidermal layer which was indistinguishable from the control mice (Fig 3.1.A). This is in contrast to the lower lip in which active infection was occurring (Egan et al, 2016). The lower lips from infected mice had increased inflammatory cells retained in the dermal layer. Immunohistochemistry was performed on the lip tissue sections and confirmed that no viral antigen could be identified. Antibodies specific for the T cell marker CD3 confirmed that a large number of the retained cells were T lymphocytes. Additional antibody labeling demonstrated that the majority of the cells were $CD3^+$ $CD4^+$ T cells which were found in the dermal layer of the lip. $CD3^+$ $CD8^+$ T cells could be could be found in the epidermal layer of the lip. We observed that $CD3^+$, $CD4^+$, and $CD8^+$ T cells could be identified in hair follicles in the lip (Fig 3.1.B). These results are consistent with previous reports that virus specific T cells were retained in peripheral tissue where the original infection occurred (Mackay et al., 2012).

Figure 3.1. Mice do not exhibit pathology in the lip 30 days post-infection. Mice were inoculated with 6.0×10^5 PFU HSV-1 McKrae and allowed to recover for 30 days. The lower lip was processed for histological sectioning and staining. (A) Hematoxylin & Eosin staining of the lower lip. (B) Tissue sections were treated with antibodies specific for HSV-1 (green) and CD3, CD4, or CD8 (red) and counterstained with DAPI (blue). White arrowheads point to antibody positive cells which are located within hair follicles. One representative experiment is shown with n=3 mice.

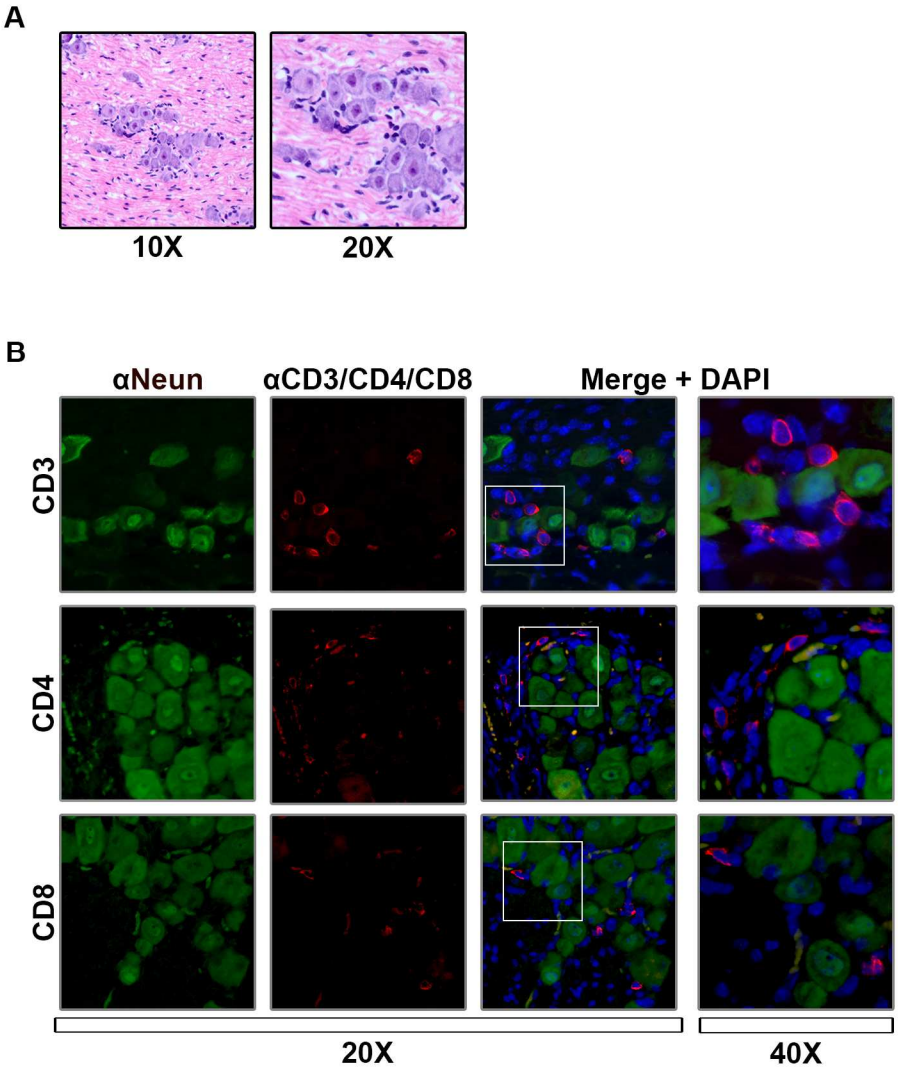
Figure 3.1.


A
10X
20X
B
αHSV-1
αCD3/CD4/CD8
Merge+DAPI
Merge+DAPI
CD3
CD4
CD8

A hallmark of HSV-1 latency in animal systems is the infiltration into and retention of T lymphocytes within the TG. These cells begin infiltrating the TG during acute infection, then associate with and surround virus-infected neurons. After a contraction phase of the immune response, a reduced but still present population of virus-specific T cells is retained in the TG of the mouse (Fig. 3.2.A). Immunohistochemistry was performed using antibodies directed against CD3, CD4, and CD8 to confirm that these cells were retained in the TG 30 days post-infection. Few T cells were still shown to be resident in the TG. Additional antibody labeling demonstrated that both $CD4^+$ and $CD8^+$ were still present in the TG during latency (Fig 3.2.B.). Confirmation that $CD4^+$ and $CD8^+$ T cells were identified in the TG was an important finding which provides additional evidence that infection resulting from topical administration of virus following lip scarification leads to retained lymphocytes in the TG similar to what has been reported in other routes of infection (Khanna et al., 2003).

Figure 3.2. $CD4^+$ and $CD8^+$ T cells are retained in the latently infected trigeminal ganglia. Mice were inoculated with 6.0×10^5 PFU HSV-1 McKrae and allowed to recover for 30 days. The TG was processed for histological sectioning and staining. (A) Hematoxylin and Eosin staining of the TG 30 days post-infection. (B) Tissue sections were treated with antibodies against Neun (green) and CD3, CD4, or CD8 (red) and counterstained with DAPI (blue). White boxes indicate regions of interest which are shown in higher power panels on the right. One representative experiment with n=3 mice.

Figure 3.2.



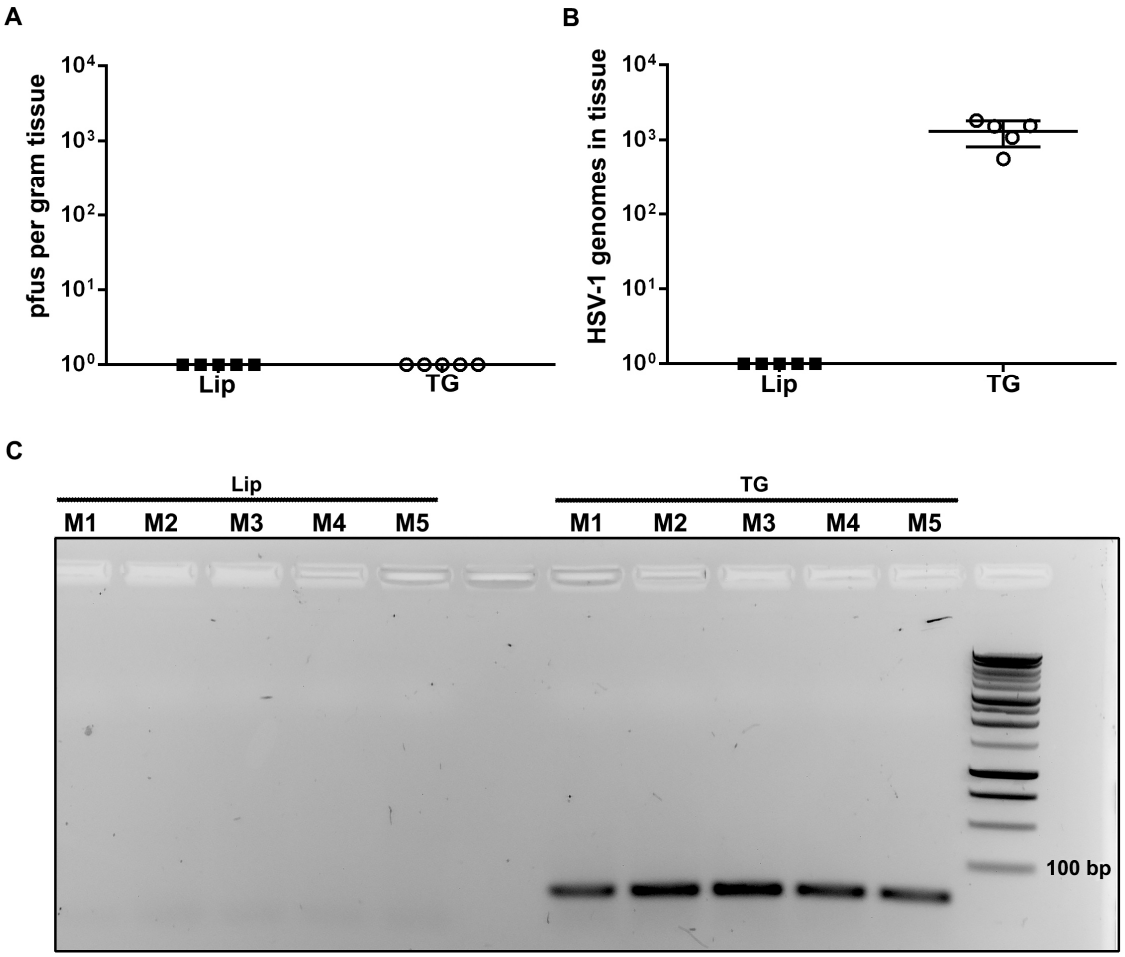
3.4.2. Latent HSV-1 genomes are retained in the TG

Latency from animal models is demonstrated by a lack of replicating virus present in the periphery and TG from infected animals. The lip and TG from mice sacrificed 30 days post-infection was analyzed for presence of replicating virus by standard plaque assay. We were unable to detect any replicating virus in the lip or TG from mice which were inoculated with HSV-1 McKrae (Fig. 3.3.A).

We next determined if the lip or TG from infected mice retained HSV-1 genomes. The lip and TG from mice sacrificed 30 days post-infection were harvested and genomic DNA isolated. PCR amplification of the thymidine kinase (TK) gene demonstrated that there were indeed HSV-1 genomes retained within the TG. We detected 1.3×10^3 HSV-1 genomes in the TG, which was consistent with our previous report of HSV-1 genomes present in the genomes at 15 days post-infection (Fig. 3.3.B). In contrast, no viral genome copies were detected in the lip 30 days after infection. The RNA from mice sacrificed 30 days after infection was used to detect LAT expression in the TGs from mice. In this regard, LAT expression was detected in all TGs isolated from infected mice but no LAT could be detected in the lip samples (Fig. 3.3.C.).

Figure 3.3. The trigeminal ganglia contains viral genome copies 30 days post-infection. Mice were infected with 6.0×10^5 PFU HSV-1 McKrae. The mice were sacrificed 30 days post-infection, and the lip and TG were isolated. Plaque assays were performed on cell-free homogenates from the lip and TG. PFU values were made relative to the mass of tissue analyzed (A). Viral genome copies present in the lip and TG were determined by qPCR . Genome copy values were made relative to the mass of the tissue analyzed (B). Total RNA was isolated from the lip and TG and reverse transcribed. PCR was performed using primers specific for the major LAT species (C). One representative experiment is shown with n=5 mice.

Figure 3.3.


A
pfus per gram tissue
10^4
10^3
10^2
10^1
10^0
Lip
TG
B
HSV-1 genomes in tissue
10^4
10^3
10^2
10^1
10^0
Lip
TG
C
Lip
TG
M1
M2
M3
M4
M5
M1
M2
M3
M4
M5
100 bp

These results provide compelling evidence that latency can be established in mice inoculated via the lip scarification method. No pathology or viral antigen was shown to be present in the lip 30 days after infection. There are retained lymphocytes in the lip from infected mice where the original infection occurred, and these could play a role in limiting replication of future infections in that area. We demonstrated that the TG was infiltrated with $CD3^+$ $CD4^+$ and $CD8^+$ T cells beginning during the acute infection and that these are retained through 30 days after infection. There was no infectious replicating virus present in the lip or TG at 30 days post-infection. The number of genome copies present in the TG was consistent with levels present in the TG 15 days post-infection. We did not detect LAT expression in the lip tissue but LAT expression was consistently detected in the TGs. The bands observed were of an appropriate size with our predicted amplicon size of 85 bp.

3.4.3. Ex vivo culture of TGs induces reactivation

An important feature of any model of HSV-1 latency is the capability of the virus to reactivate. To study this process, TGs from mice sacrificed 30 days post-infection with Vero cells were cocultured with permissive indicator cells. To this end, Liberase TM digestive cocktail was used to disperse surgically removed TGs into single cell suspensions. The digested TGs were apportioned onto monolayers of Vero cells, covered with growth media, and incubated for 10 days to allow infected cells to reactivate. The media from these cultures was sampled daily and used in standard plaque assays to determine when infectious virions had been produced.

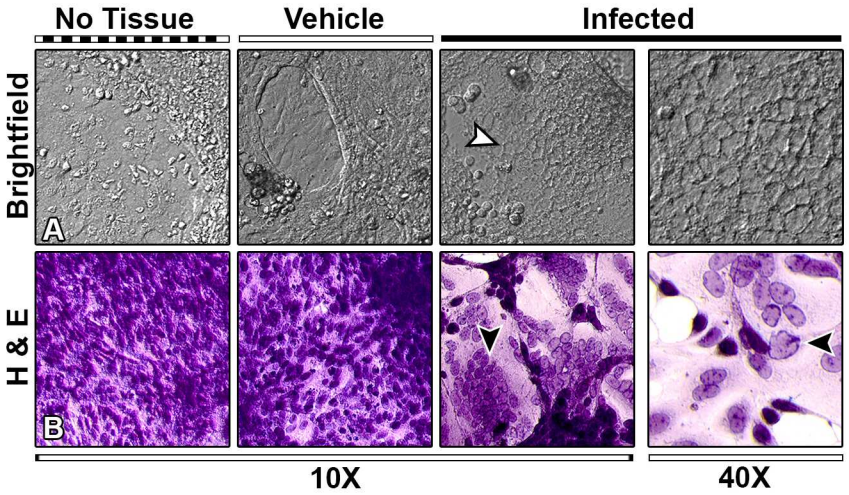
We observed infectious virions present in the culture media from the TGs between 4 and 6 days after plating (Table 3.1). At the end of the 10 day incubation period, the monolayers displayed a clear cytopathic effect consistent with HSV-1 infection (Fig. 3.4.A) Compared to tissue from uninfected mice, the monolayers had multinucleated cell bodies which is evidence of cell fusion from neighboring infected cells. Plaque morphology staining in the primary cocultures revealed the fused multinucleated bodies and evidence of chromatin margination of infected cells (Fig. 3.4.B). Chromatin margination is a well-known viral inclusion body associated with HSV-1 infections and can be observed in HSV-1 infected tissue and cell cultures (Leinweber et al., 2006). Similar chromatin margination was observed in our plaque morphology staining from stock HSV-1 strains.

Table 3.1. Cultured lip and trigeminal ganglia tissue reactivate from latency

Mouse	Treatment	Reactivation	Day of TG reactivation	Day of lip reactivation
1	Vehicle	No	ND	ND
2	Vehicle	No	ND	ND
3	Vehicle	No	ND	ND
4	Vehicle	No	ND	ND
5	HSV-1	Yes	4	5
6	HSV-1	Yes	4	7
7	HSV-1	Yes	6	7

Figure 3.4. Cocultures of trigeminal ganglia from infected mice exhibit cytopathic effect. The TGs from uninfected and infected mice 30 days after inoculation were enzymatically digested and plated on Vero cells. After 10 days in culture, the plates were fixed. (A) Brightfield imaging of cultures of Vero cells only (no tissue), cocultures with uninfected TGs (vehicle) and infected TGs (infected). White arrowhead points to an example of an area of cytopathic effect in the monolayer. (B) Plates were stained with Hematoxylin and Eosin dyes and imaged. Black arrowheads point to HSV-1 inclusion bodies.

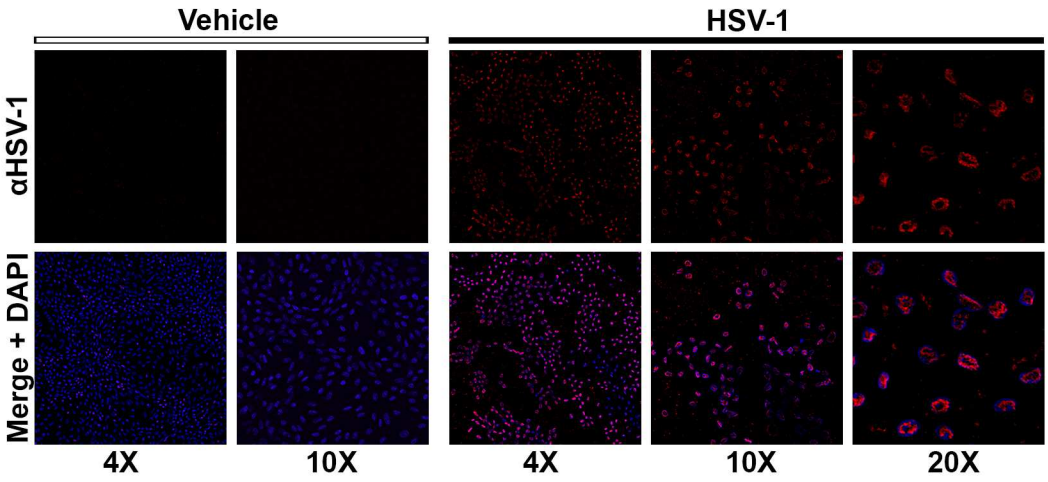
Figure 3.4.



In order to confirm that the apparent cytopathic effect in the primary tissue monolayers was due to HSV-1, the sampled media was used to infect Vero cell monolayers and perform immunocytochemistry. Using polyclonal antibodies specific for HSV-1 antigen, HSV-1-positive cultures were detected from media derived from infected tissue coculture and HSV-1-negative cultures were obtained from media sampled from uninfected tissue coculture (Fig. 3.5).

Figure 3.5. Sampled media from trigeminal ganglia cocultures contain infectious HSV-1. The media from cocultured TGs from uninfected and infected mice was sampled daily for presence of infectious HSV-1. The sampled media was plated over monolayers of permissive Vero cells. The cells were fixed after 48 hours and treated with antibodies specific for HSV-1 (red) and counterstained with DAPI (blue).

Figure 3.5.



3.4.4. Ex vivo cultivation of infected lips induces virus reactivation

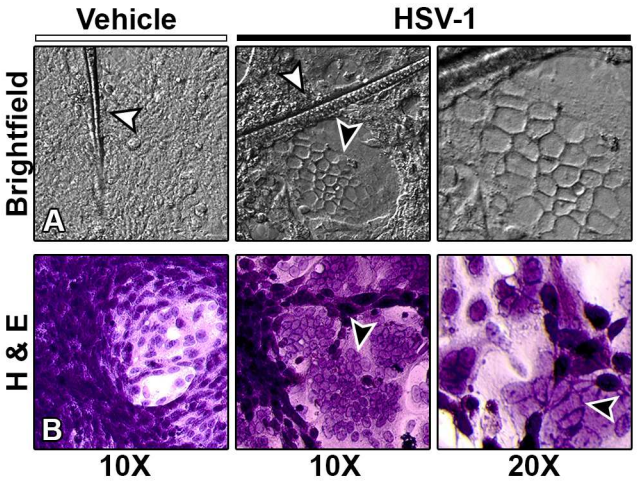
Previously we reported on the pathological and immunological events that occur during the acute phase of infection with HSV-1 using the lip scarification model. We observed that although there was a dramatic reduction in viral replication and genome copies between 8 and 15 days post-infection, there were still detectable genomes present in the lip. This result was interesting and led us to determine if this could be an example of established peripheral latency. Although we were unable to detect genome copies or LAT expression in the lip 30 days post-infection, it was possible that the established latent infection was below the level of detection from these methodologies. To determine if it was possible that a peripheral latent infection has been established, enzymatically digested lip tissue was cocultured with Vero cells for 10 days. Even a very low amount of latently infected cells below the level of detection by PCR would be expected to reactivate and be detectable in the indicator Vero cells.

The lips from mice infected with HSV-1 or treated with vehicle were surgically removed from sacrificed mice 30 days after infection. The lips were minced and digested using a cocktail of collagenase and DNAse1. The digested lips were dispersed and plated evenly on Vero cells and incubated for 10 days at 37°C in the presence of 5% CO_2. The media was sampled daily and used for plaque assay analysis for detection of produced infectious virions. At the end of 10 days, the plates were fixed and imaged with brightfield microscopy before plaque morphological staining.

At the end of the 10 days, the monolayers exhibited cytopathic effect similar to those observed with the reactivated TG cultures. Clear areas of CPE could be distinguished from uninfected neighboring cells and were not observed in Vero cells with the digested lip from uninfected mice (Fig. 3.6.A). After staining with Hematoxylin and Eosin, the Vero cell monolayers displayed clear multinucleated cell bodies and chromatin margination (Fig. 3.6.B).

Figure 3.6. Cocultures of lip from infected mice exhibit cytopathic effect. The lips from uninfected or infected mice 30 days after inoculation were enzymatically digested and plated on Vero cells. After 10 days in culture, the plates were fixed. (A) Brightfield imaging of cultures of Vero cells, cocultures with uninfected lips and infected lips. White arrowhead points to areas of cytopathic effect in the monolayer. (B) Plates were stained with Hematoxylin and Eosin and imaged. White arrowheads point to hair shafts from mice present in the culture. Black arrowheads point to cytopathic effect and HSV-1 inclusion bodies.

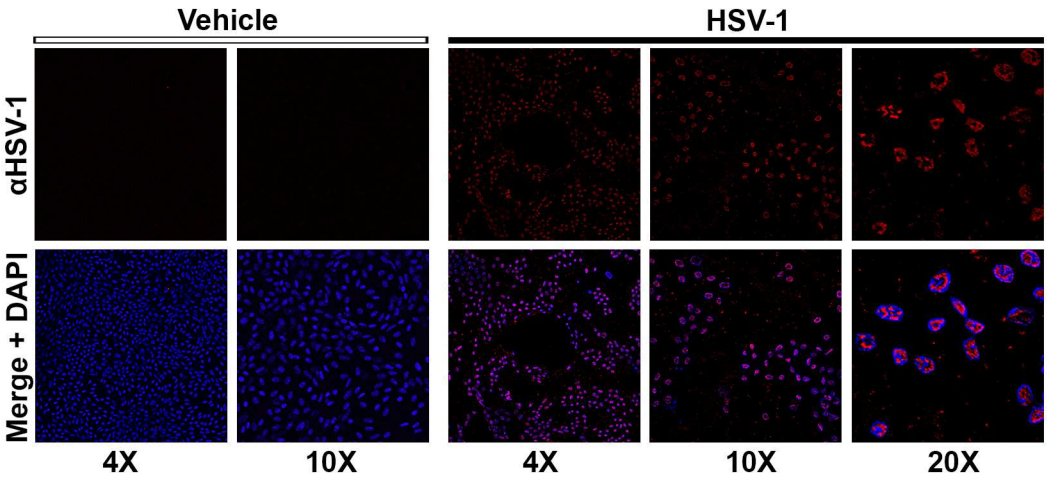
Figure 3.6.



The sampled media from infected lip cultures was used for infecting new monolayers of Vero cells and performing immunocytochemistry. Using a polyclonal antibody specific for HSV-1 antigen, we confirmed that the plaques produced were from HSV-1 virions contained within the sampled media of reactivated explant lip cultures (Fig. 3.7).

Figure 3.7. Sampled media from trigeminal ganglia cocultures contain infectious HSV-1. The media from coculturing lips from uninfected and infected mice was sampled daily for presence of infectious HSV-1. The sampled media was plated over monolayers of Vero cells. The cells were fixed after 48 hours and treated with antibodies specific for HSV-1 and counterstained with DAPI.

Figure 3.7



3.4.5. Isolated virus produces plaques with consistent morphology to stock HSV-1 McKrae

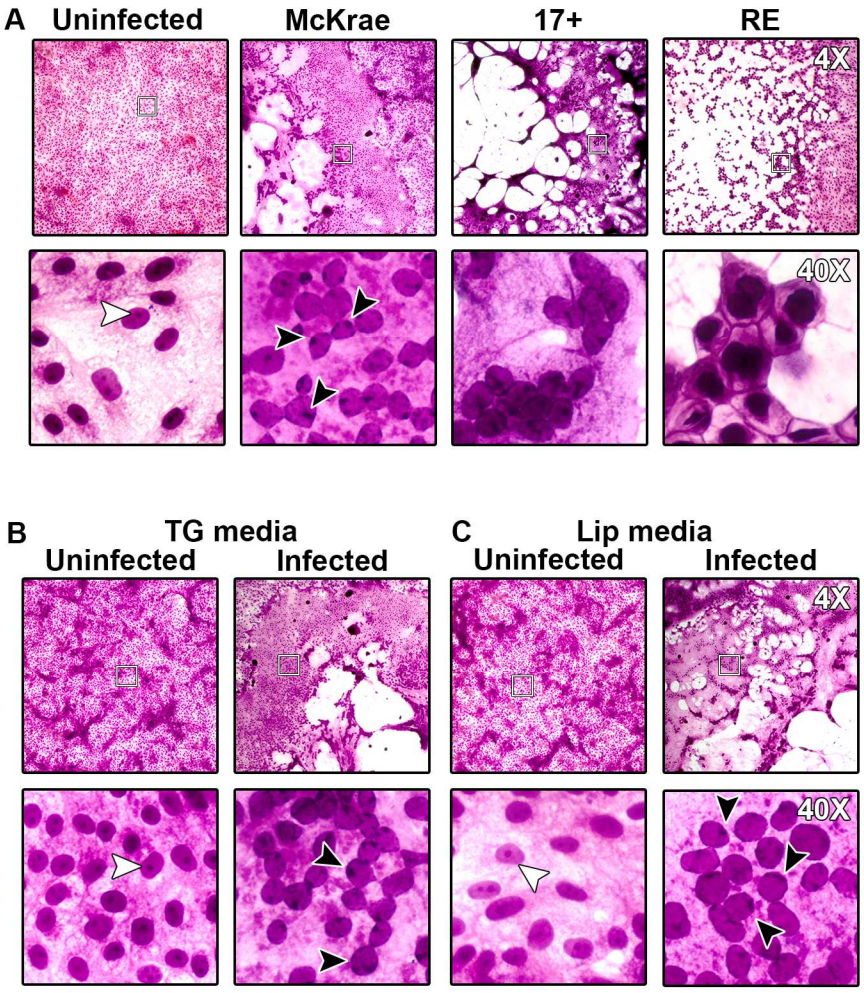
Finally to confirm that the reactivated HSV-1 was similar to the initial stock virus used to infect animals, a plaque morphology staining procedure was developed. This method, using standard histological Hematoxylin and Eosin dyes, was able to reveal great detail in differences in the plaques formed from different stock HSV-1 strains. Uninfected Vero cells exhibited plump cylindrical nuclei with prominent nucleoli. The stock HSV-1 McKrae strain revealed large areas of infected and dying cells that surrounded the cleared areas in the center of the plaques. The very center of the plaques contained cleared areas where cell death had already occurred. In addition, the center of the plaques contained large multinucleated cell bodies. The outer zone of infection contained many singular cells which were infected but still contained viral inclusion bodies such as chromatin margination. The stock 17^{+} strain of HSV-1 (Brown et al., 1973) exhibited a similar large zone of infection surrounding the central zone of clearance of the plaque. However, the 17^{+} strain had numerous multinucleated cell bodies in the outer infection zone. The stock RE (Irvine et al., 1967) strain exhibited a very distinct plaque morphology with a very large zone of clearance in the center and the outer zone containing fused cells with 1-2 nuclei each and a prominent cell body border with neighboring fused cells (Fig. 3.8.A)

The sampled media from the infected lip and TG cultures produced plaques consistent with the stock HSV-1 McKrae strain. There were large zones of infected cells surrounding a central zone of clearance which also contained

multinucleated cell bodies. The cells in the outer zone of infection were smaller and more round than the plump cylindrical cell bodies from uninfected cultures. Additionally, the cells in the outer zone of infection contained viral inclusion bodies including chromatin margination. The morphology of the plaques were most consistent with the morphology of the plaques produced by the stock HSV-1 McKrae strain and not the other stock HSV-1 strains examined (Fig. 3.8.B and 3.8.C).

Figure 3.8. HSV-1 from sampled media of lip and TG cultures has similar plaque morphology to the Mckrae strain. (A) Vero cells were infected with different clinical isolates of HSV-1 and stained with Hematoxylin and Eosin. (B) Media sampled from TG cultures of uninfected (left) and infected (right) mice was plated on Vero cells. The cells were then fixed and stained with Hematoxylin and Eosin. (C) Media sampled from lip cultures of uninfected (left) and infected (right) mice was plated on Vero cells. The cells were then fixed and stained with Hematoxylin and Eosin. White arrowheads point to prominent nucleoli in uninfected Vero cells. Black arrowheads point to chromatin margination in infected Vero cells.

Figure 3.8.


A
Uninfected
McKrae
17+
RE
4X
40X
B
TG media
Uninfected
Infected
C
Lip media
Uninfected
Infected
4X
40X

3.5. DISCUSSION

We previously reported that the lip scarification model of infection was a physiologically relevant model for studying human HSV-1 infections in the orofacial tissue. Our model uses the HSV-1 McKrae as the infectious viral strain because it is particularly neurovirulent in animal models (Hill et al., 1987). We combined this neurovirulent model with the adult C57BL/6 mouse model because it is resistant to encephalitis caused by neurovirulent strains of HSV-1 (Kastrukoff et al., 2012). The inoculation route through the lip is particularly relevant to human infections but also minimizes the risk of encephalitis because of the greater distance that needs to be traveled to gain access to the TG and CNS. Taken together, this model has the potential to be of great value in studying HSV-1 infections in humans, establishment of viral latency, and HSV-1 reactivation. Previously, we reported the pathological and immunological events that occur during the acute phases of infection. Here we report on the latent time points of infection following lip scarification and inoculation with HSV-1 McKrae.

We confirmed that there was no pathology present in the lip consistent with the absence of replicating virus in mice which were productively infected 30 days prior. By 30 days after infection, the tissue architecture had returned to normal with a thin epidermis but increased cellularity. Immunohistochemistry assays revealed numerous $CD3^+$, $CD4^+$, and $CD8^+$ T lymphocytes present in the lip (Fig 3.1). This is consistent with observations obtained with other models which demonstrate that memory T-cell populations reside in tissue sites where antigen was first encountered. These tissue resident memory T (T_{RM}) cells were

retained in tissue locations where they can immediately respond to antigen previously seen and initiate memory responses. TRM cells have been found in the skin of mice inoculated with HSV-1 through the flank skin (Mackay et al., 2012), and are suspected to be in TG of mice inoculated with HSV-1 through the ocular route (Himmelein et al., 2011; Khanna et al., 2003).

Animal models and post mortem tissue has consistently shown T cells to be retained in the TG after exposure to HSV-1 at a time postinfection at which no replicating HSV-1 virions can be detected. We used antibodies specific for $CD3^+$, $CD4^+$, and $CD8^+$ T cells to confirm that these cells are retained in the TG of mice infected via lip scarification. As shown, we were able to detect them in tissue from mice 30 days after infection (Fig 3.2). The numbers of T cells present in the tissue 30 days after infection were low, but present in multiple sections and from all mice examined. It was possible that altering the dosage of virus would lead to a greater number of T cells retained in the TG. Because the C57BL/6 mice are resistant to encephalitis and the distal site of inoculation to the CNS, we can experiment with higher doses of virus to observe differences in immune responses.

A standard time point for examining latency in models was to look 28 days after infection. We believe that after 30 days, our mice have successfully established latency. We do not detect any replicating virus in cell-free homogenates in the lip or TG by plaque assay of freshly isolated tissue. PCR amplification of the TK gene demonstrates that viral genome copies are still maintained in the TG even though no replicating virus was detected. PCR

amplification of the major LAT RNA species shows that the LAT gene was being expressed in the latently infected TGs (Fig 3.3). Lack of replicating virus, presence of HSV-1 genomes, and expression of the LAT gene are all hallmarks of a successful latent infection from *in vivo* models.

Surgical removal of latently infected tissue and coculture with indicator cells is a well known method used to induce reactivation from tissue. Instead of coculturing the whole tissue, the tissue was enzymatically digested to form a single cell suspension before plating. All of the TGs from mice which were infected reactivated after a delay between 4-6 days in culture (Table 3.1). This indicated that it was not due to the presence of undetectable infectious virus present in the tissue. The stress of sacrifice and surgical removal of tissue induced reactivation in latently infected cells. But the reactivation transcriptional program needs time before new infectious virions can be produced. The time delay between sacrifice and presence of infectious virions in a strong indicator that reactivation from latently infected cells has occurred. In order to confirm the transcriptional program has been initiated, a careful time course experiment must be done to detect presence of lytic transcripts to determine if a clear shift in patterns can be detected. The dispersal to single cell suspensions will allow the measurement of how many cells have been latently infected by infectious center assay.

A surprising result was the presence of infectious virus in the media from the lip cultures. During tissue harvest, the lips were the first tissue removed. The time from animal sacrifice to removal of the lip is approximately 5 minutes. This

time window was too short for reactivation to be initiated in the TG and travel to the lip. This has indicated that HSV-1 found in lip cultures derived from a persistent low level infection in the lip or from reactivation from a peripheral latent site. We did not detect any infectious HSV-1 virions from freshly isolated lips by plaque assay of cell-free homogenates. These results suggest that the presence of infectious HSV-1 virions was likely due to reactivation from a latent state. HSV-1 genomes were not detected in the lip by qPCR nor was LAT expression detected in the lip. It is possible if a latent infection is maintained in the lip, it was in such low levels that it was not detectable by these techniques. The media sampled from the lip cultures contained HSV-1 virions as confirmed by immunocytochemistry using antibodies specific for HSV-1 antigen. Additionally the plaque morphology staining confirmed that the HSV-1 was consistent with the input stock of HSV-1 McKrae strain. These results suggest that reactivation has occurred in lip cultures and the virus produced was HSV-1 and from the McKrae strain which was used to inoculate the mice.

Peripheral latency has been a controversial idea in the field of HSV-1 latency. Peripheral reactivation has been reported in the clinical setting of corneal transplantation (Higaki et al., 2015; Kaye et al., 1991). Some mouse studies have demonstrated that HSV-1 can be recovered from culturing mouse corneas (Abghari and Stulting, 1988). Additionally it was reported that the appearance of HSV-1 was delayed in the corneas compared to matched trigemina ganglia. However, there are similar mouse studies which fail to recover HSV-1 from latently infected mice (Easty et al., 1987). It appeared that the flexibility in mouse

strains along with different HSV-1 strains has led to an unexpected difficulty in comparing results from different mouse and HSV-1 strains. In order for this question to truly be addressed in mice, a singular mouse strain matched with a singular strain of HSV-1 should be used consistently in multiple experiments. We feel that the lip scarification inoculation method using C57BL/6 mice and HSV-1 McKrae is a suitable model to study peripheral latency in the mouse lip.

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Chapter IV

Delivery of CRISPR/Cas9 gRNAs targeting HSV-1 immediate early genes using AAV vectors

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Kevin Egan conceptualized experiments, performed experiments, collected and analyzed data and wrote the chapter. Alexander Allen collected data. Drs. Stephen Jennings and Brian Wigdahl participated in the intellectual development, conceptualized experiments and critically evaluated all aspects of the chapter. Dr. Stephen Jennings is supported by developmental funds provided by the Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine

4.1.ABSTRACT

Herpes simplex virus type 1 (HSV-1) infections persist for the lifetime of the host. Current therapies are not curative as they do nothing to remove the latent genome of the virus or affect its ability to reactivate. CRISPR/Cas9 gene editing is a novel technique which targets the viral genome and has the potential to be uniquely curative. Guide RNAs (gRNAs) targeting HSV-1 immediate-early genes ICP0 and ICP27 were generated in an adeno-associated virus (AAV) vector system. We injected these AAV vectors into the lower lip of mice and evaluated expression of the gRNAs in the lip and TG. We observed maximal expression of gRNAs in the lip and TG 24 hours after injection. A high dose of AAV had greater expression of gRNAs in the lip compared to a low dose. However the low dose of AAV had greater expression in the TG compared to high dose. These studies demonstrate that CRISPR/Cas9 AAV vectors can be delivered locally and express gRNAs in peripheral tissue as well as the TG.

4.2. Introduction

Herpes simplex virus type 1 (HSV-1) establishes a latent infection in humans which persists for the life time of the host. The acute infection is most often resolved without lasting pathology. But the virus remains in a latent state within the neurons innervating the initial site of infection, capable of reactivating periodically and causing recurrent disease. Gene editing technologies hold great promise for being a possible curative therapy for latent HSV-1 infections. Targeting HSV-1 genes in the latent episomal genome could prevent the virus from reactivating or even possibly eliminate the genome from infected neurons. Clustered regularly interspaced short palindromic repeat (CRISPR) Cas9 is a novel gene editing technology which uses guide RNAs to target DNA sequences for cleavage.

During lytic replication of HSV-1, the viral genome exists as concatameric double-stranded DNA that is later cleaved to form singular units of the linear double-stranded DNA genome (Muylaert et al., 2011). In contrast to this, the latent HSV-1 genome is maintained as a circular episome that is bound by histones bearing repressive heterochromatin regions (Efstathiou et al., 1986b; Rock and Fraser, 1983). Viral transcription during latency is repressed and the only viral gene found to be abundantly expressed is the latency-associated transcript (LAT) (Javier et al., 1988; Wagner et al., 1988; Wagner and Bloom, 1997). There is leaky transcription of other viral lytic genes, but they do not appear to lead to translation of viral proteins in appreciable numbers and amounts. When reactivation is initiated in response to specific triggers, viral lytic

gene transcription initiates and the virus undergoes productive replication in the neuron. Newly formed HSV-1 virions (or potentially capsids) traffic down the axon of the innervating sensory neuron and are released into the periphery where the axonal termini are located. The released virions undergo productive infection in the peripheral site with associated pathology of reactivation events.

Current antiviral therapies available consist of nucleoside analogs (Villa et al., 2016). Acyclovir and it's derivatives are preferentially phosphorylated by the viral-encoded thymidine kinase and the triphosphorylated compounds are preferentially incorporated into the elongating HSV-1 genome as a result of the activity of the HSV-1-encoded DNA polymerase (Birkmann and Zimmermann, 2016). The triphosphorylated nucleotide analogs lack the requisite ribose sugar and hydroxyl group for addition of new nucleotides to the analog and therefore halt elongation of the viral genome after their incorporation and the viral replication process is subsequently halted. Although these therapeutics have proven to be highly effective in treating primary and recurrent infections, they only act on actively replicating virus. These therapeutics have no activity on the latent HSV-1 genome and thus do not constitute a curative therapy. In order to prevent future reactivations, a person must always be on a course of chain termination therapy. A potential curative therapy must have the ability to target the latent HSV-1 genome for irreversible inactivation or complete removal of the latent genome.

CRISPR-Cas9 gene-editing is a novel technique which has the potential for such targeting. CRISPR/Cas9 was originally discovered as an ancient antiviral response in bacteria (Barrangou et al., 2007). The Cas9 protein acts as a nuclease to introduce cleavages in DNA sequences (Ehrlich et al., 2005; Makarova et al., 2006; Marraffini and Sontheimer, 2008). The Cas9 protein is guided to target DNA by guide RNAs (gRNAs) which hybridize to the targeted DNA sequence (Jinek et al., 2012). The target DNA sequences must be immediately followed by a Protospacer adjacent motif (PAM) sequence for the Cas9 protein to cleave the target DNA. The promise of the CRISPR/Cas9 technique is that as long as a DNA sequence contains PAM sequences, then gRNAs can be designed which will direct the Cas9 protein to cut at that site. This technique is already being explored for gene therapy use in humans for correcting heritable disorders arising by single nucleotide polymorphisms (SNPs) (Chang et al., 2015).

A delivery of Cas9 protein and gRNAs to cells harboring latent HSV-1 genomes could lead to efficient targeting of the viral genome for editing. Targeting of genes required for replication could inhibit the virus from being able to reactivate from latency. Recently, investigators have begun to experiment with gRNAs targeting HSV-1 genes using in vitro experiment model systems. Stable Vero cell lines which express gRNAs targeting a variety of HSV-1 genes have shown efficacy in limiting HSV-1 replication. Most gRNAs targeting HSV-1 genes were ineffective at limiting replication 3 days post-infection, but gRNAs targeting the primase-helicase complex remained inhibitory (van Diemen et al., 2016).

Plasmids encoding Cas9 and gRNAs targeting the HSV-1 immediate- early genes ICP0, ICP4, and ICP27 have been generated and tested in vitro. These experiments demonstrated that HSV-1 infection was limited when cells were treated with gRNAs. Additionally, it was found that the HSV-1 genome had insertions/deletions caused by Cas9 nuclease activity in targeted sequences. Finally, the designed gRNAs were found to have minimal off-target effects (Roehm et al., 2016).

In this chapter, we treated mice with AAVs encoding gRNAs which target the HSV-1 immediate early genes ICP0 and ICP27. We wanted to evaluate if AAV delivered gRNAs are expressed in the lip and the TG. We used a local delivery of AAVs by injecting directly into the lower lip of anesthetized animals. We found that the gRNAs are maximally expressed 24 hours after injection. An important finding was that AAVs delivered to the lower lip were capable of making it to and express the gRNAs in the TG.

4.3. MATERIALS AND METHODS

4.3.1. Mice

Wild-type C57BL/6 adult (procured at 12 weeks of age) male mice were purchased from Jackson Laboratories. All animal experiments adhered to procedures and guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Drexel University. Mice were anesthetized with Avertin and AAV vectors were injected directly into the lower lip in a volume of 10 µl.

4.3.2. Design of CRISPR gRNAs targeting HSV-1 genes

Preparation of CRISPR gRNAs targeting the second exon of ICP0 was performed as previously described (Roehm et al., 2016). Briefly, the sequence of HSV-1 ICP0 was obtained (NC_0018061) and gRNA targets were designed using available online tools by Kamel Khalili at Temple University. gRNAs targeting the HSV-1 ICP4 and ICP27 genes were also generated from available databases and online tools. The gRNAs were cloned into pX260 plasmid vectors with a Cas9 gene. Plasmid vectors encoding Cas9 and the gRNA targeting the appropriate immediate-early gene (ICP0, ICP4, or ICP27) were grown up using DH5 alpha bacteria and the plasmid isolated using the plasmid Maxi procedure (Qiagen) as previously described by the manufacturer. The DNA concentration was obtained by spectrophotometry.

4.3.3. Mouse treatment with AAV gRNA vectors

AAV vectors encoding Cas9 and gRNAs targeting ICP0 or ICP27 were kindly provided from Kamel Khalili at Temple University. A cocktail of AAV encoding gRNAs targeting ICP0 and ICP27 at a concentration of 4.3×10^{10} PFUs or

$2.\times10^{10}$ PFUs respectively in a final volume of 10 µl (1X) was injected into the lower lip of anesthetized mice. We also used a cocktail of AAVs encoding ICP0 and ICP27 gRNAs at a concentration of 21.5×10^{11} and 1.4×10^{11} PFUs respectively in a volume of 10 µl (5X). Mice were allowed to recover and then 3 mice were sacrificed at indicated times. The lower lips and TGs were harvested and frozen in Dulbeccos Modified Eagles Medium (DMEM).

4.3.4. Evaluation of gRNA expression in tissue

The RNA from mouse tissue was isolated using TRIzol® Reagent. The tissues were homogenized using a Bullet Blender™ with zirconium oxide beads. The homogenate was incubated with TRIzol® Reagent and the RNA isolated using a pheno chloroform procedure per manufacturer's protocol.

4.3.5.Statistics

Data were graphed using GraphPad Prism version 6.0.

4.4. RESULTS

4.4.1 ICP0 gRNAs are maximally expressed in the lip 24 hours after injection

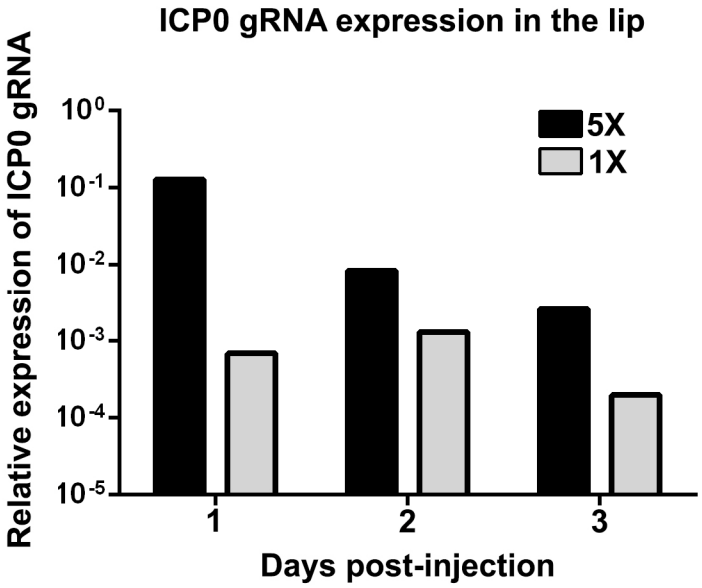
CRISPR/Cas9 mediated gene therapy has a great potential to be a therapeutic which can act on latent HSV-1 genomes. There are numerous vectors which can serve to deliver DNA sequences encoding Cas9 and targeted gRNAs to cells *in vivo*. AAV vectors are already being used for gene therapy in humans and have been used to delivery CRISPR/Cas9 sequences in mice. We employed AAV vectors encoding Cas9 and gRNAs which target the HSV-1 immediate early

genes ICP0 and ICP27. We delivered a cocktail of AAVs targeting both HSV-1 genes through direct injection into the lower lip. We tested two different doses of the AAVs (1X) and (5X) as described in the methods. Mice were sacrificed at indicated time points and the lip and TG were harvested. We evaluated the lip and TG from treated mice for expression of gRNAs targeting HSV-1 genes.

We found that AAV delivered gRNAs targeting ICP0 were maximally expressed 24 hours after injection. The highest expression was seen in mice treated with the 5X dose of the AAV vectors. After 24 hours, the expression levels of the gRNAs decreased through 3 days post-injection. The 1X dose did not have the same level of decrease, but was 2 logs reduced in relative expression level compared to the 5X dose (Fig 4.1.).

Figure 4.1. AAV delivered gRNAs targeting ICP0 are maximally expressed 24 hours post-injection. AAVs encoding gRNAs which target ICP0 were directly injected into the lower lip of mice. The mice were allowed to recover and at indicated time points, were sacrificed. The lower lip was harvested and expression levels for the gRNAs was determined. Expression levels are relative to the house keeping gene beta actin.

Figure 4.1.

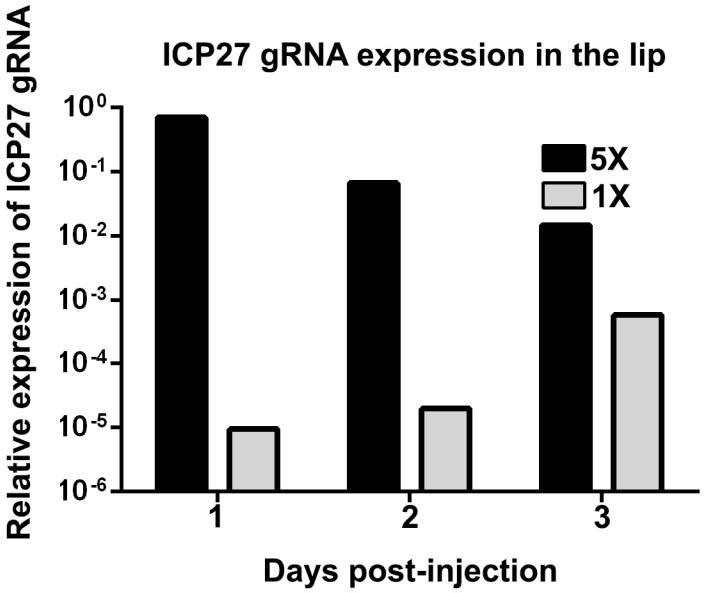

ICP0 gRNA expression in the lip
Relative expression of ICP0 gRNA
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
5X
1X
1
2
3
Days post-injection

4.4.2. Expression of ICP27 gRNAs in the lip is highest 24 hours after injection.

We next evaluated if the AAV delivered gRNAs which target ICP27 are expressed in the lip after injection. We found that these gRNAs also have the highest relative expression in the lip 24 hours after injection. The expression decreased as the length of time after injection increased. Similar to the ICP0 gRNAs, the 5X dose of AAVs had a much greater relative expression of gRNAs compared to the 1X dose of gRNAs in the lip. One interesting result is that the 1X dose of AAVs seemed to increase in expression at later time points, but the level of expression of the 1X gRNAs did not approach the expression level seen with the 5X dose during the time period examined (Fig 4.2).

Figure 4.2. Expression of ICP27 gRNAs in the lip is highest 24 hours after injection. AAVs encoding gRNAs which target ICP0 were directly injected into the lower lip of mice. The mice were allowed to recover and at indicated time points, were sacrificed. The lower lip was harvested and expression levels for the gRNAs were determined. Expression levels are relative to the house keeping gene beta actin.

Figure 4.2.


ICP27 gRNA expression in the lip
Relative expression of ICP27 gRNA
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
5X
1X
1
2
3
Days post-injection

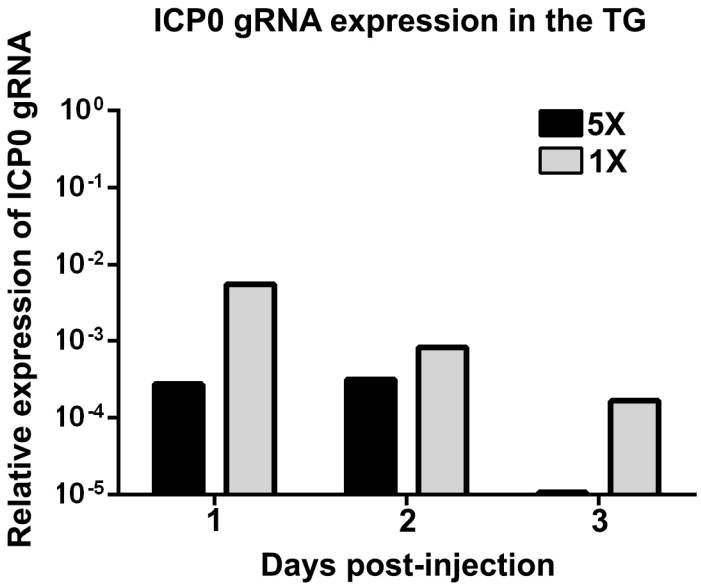
4.4.3. ICP0 gRNA expression in the TG is highest 24 hours after injection

An important consideration when evaluating AAV delivered CRISPR/Cas9 therapeutics is tissue specific expression. Because CRISP/Cas9 gene editing can potentially be a therapeutic which acts on the latent HSV-1 genome, we need to evaluate if the AAV delivered vectors reach the TG. Previous reports have demonstrated that intraperitoneal injected AAV vectors are capable of reaching the TG. However we sought to examine if locally delivered AAVs injected into the lower lip were able to express gRNAs in the TG.

The TGs from mice treated with either the 1X or 5X dose of the AAV cocktail were evaluated for expression of gRNAs. We found that like the lip, maximal expression was observed in the TG 24 hours after injection. However a surprising finding was that the 1X dose of AAVs had greater expression in the TGs that the 5X dose. This is contrast with the data from the lip which demonstrated the opposite. We observed that expression In the TG began to decrease at time points tested after 24 hours (Fig 4.3).

Figure 4.3. ICP0 gRNA expression in the TG is highest 24 hours after injection. AAVs encoding gRNAs which target ICP0 were directly injected into the lower lip of mice. The mice were allowed to recover and at indicated time points, were sacrificed. The TG was harvested and expression levels for the gRNAs were determined. Expression levels are relative to the house keeping gene beta actin.

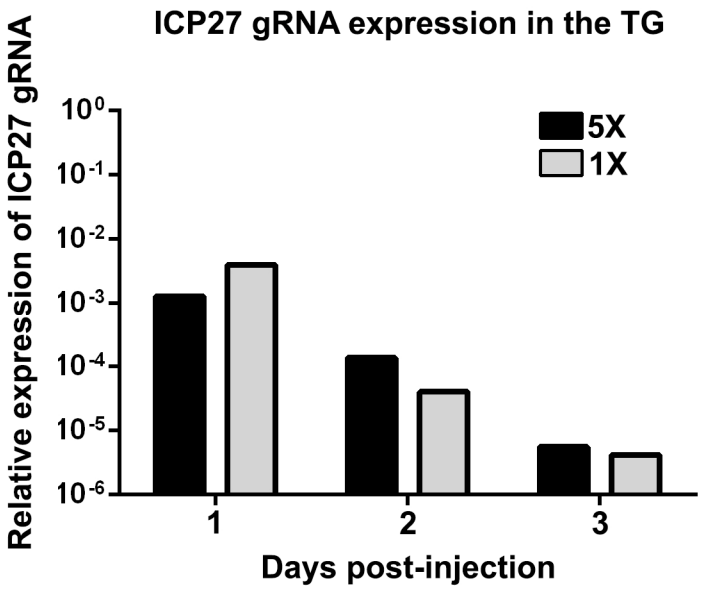
Figure 4.3.


ICP0 gRNA expression in the TG
Relative expression of ICP0 gRNA
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
5X
1X
1
2
3
Days post-injection

4.4.4. ICP27 gRNAs are maximally expressed in the TGs 24 hours after injection. The TGs from mice treated with either the 1X or 5X dose of the AAV cocktail were evaluated for expression of gRNAs. We found that like the lip, maximal expression was observed in the TG 24 hours after injection. An interesting finding is that the relative expression of the ICP27 gRNAs were comparable between the 1X and 5X doses. The 1X dose had a slightly higher relative expression, but the difference was much smaller than seen with the ICP0 gRNAs. As we have seen with the other tissues analyzed, relative expression decreased at time points analyzed after 24 hours (Fig 4.4).

Figure 4.4. ICP27 gRNAs are maximally expressed in the TGs 24 hours after injection. AAVs encoding gRNAs which target ICP27 were directly injected into the lower lip of mice. The mice were allowed to recover and at indicated time points, were sacrificed. The TG was harvested and expression levels for the gRNAs were determined. Expression levels are relative to the house keeping gene beta actin.

Figure 4.4.


ICP27 gRNA expression in the TG
Relative expression of ICP27 gRNA
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
5X
1X
1
2
3
Days post-injection

4.5. DISCUSSION

The CRISPR/Cas9 gene editing technique has the potential to be a curative therapeutic. Current antiviral therapies can only act on actively replicating virus. This means that current therapies cannot target the latent HSV-1 genome maintained in infected neurons. People who suffer reactivations can begin taking therapies when prodrome is felt and the recurrent disease limited. However the therapeutics cannot limit the potential for new reactivations. CRISPR/Cas9 is a gene editing which has the ability to act on the latent HSV-1 genome in infected neurons. Designed gRNAs will bring the Cas9 endonuclease to targeted sequences and double strand breaks introduced. The DNA repair mechanism of the cell will introduce mutations into the genomic sequence.

Delivery of CRISPR/Cas9 *in vivo* will be an important step towards making it into a therapeutic. Viral vectors including AAV vectors are currently being used in the clinic for gene therapy applications. We investigated the potential for AAV vectors to delivery CRISPR/Cas9 gRNAs to tissues of interest and express the desired genes. We injected AAVs directly into the lower lip of mice and evaluated if gRNAs are expressed in the lip and importantly in the TG. Delivery and expression in the TG will be necessary for CRISPR/Cas9 to act on the latent HSV-1 genome and be an effective curative therapy.

Mice were treated with AAV vectors encoding gRNAs targeting the HSV-1 immediate early genes ICP0 and ICP27. Mice were sacrificed at indicated time points and the tissue evaluated for expression of the gRNAs. We tested 2

different doses of AAV vectors and compared the expression of gRNAs from the different doses.

We observed that the ICP0 gRNAs had highest expression 24 hours after injection in the lip and TG. The expression levels declined at time points after 24 hours. We found that the 5X dose had the highest expression of gRNA in the lip. However in the TG, the highest expression was seen with the 1X dose. The ICP27 gRNA similarly had the highest expression 24 hours after injection in the lip and TG. The 5X dose of the ICP27 gRNA had the highest expression in the lip 24 hours after injection. Finally the 1X dose of the ICP27 had the highest expression in the TG 24 hours after injection.

The majority of the AAV vectors had maximal expression in the lip and TG 24 hours after injection. This indicates that timing of administration of the AAVs will be critical when testing CRISPR/Cas9 AAV vectors *in vivo*. Treatment with AAV vectors too early before infection will lead to decreasing expression of gRNAs before the Cas9 can encounter viral genomes. Additionally if the AAV vectors are delivered too late, then the virus has the potential to replicate to a level that the CRISPR/Cas9 won't have a significant effect. These studies will inform future experiments in regards to the timing of AAV administration and viral infection.

Another interesting result was the difference in expression observed between the different doses of AAV administered. We consistently observed that the 5X dose had the highest expression in the lip whereas the 1X dose had highest expression in the TG. It is possible that in the large tissue area of the lip,

there's a large number of susceptible cells which can be transduced by the AAV vectors. Additionally it is important to consider that AAV is transducing cells by viral infection with some related pathology and immune responses. It is possible that the large amount of AAV vectors delivered in the 5X dose is causing damage in the lip and possibly the innervating axons and limiting delivery of AAV vectors to the TG. The smaller amount of viruses delivered in the 1X dose has less damage in the lip and possibly innervating neurons which can allow for a greater number of AAV vectors being delivered to the TG.

There are still important questions that need to be addressed in future experiments. The expression and production of the Cas9 protein needs to evaluated in the lip and TG. Additionally it will be important to identify the cells which are being transduced in the TG. The studies in this paper only show that the gRNAs are bring expressed, they do not indicate that they are being expressed in neurons which have the potential to harbor latent HSV-1 genomes.

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Chapter V

Discussion, perspectives, and future directions

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Kevin Egan wrote the chapter. Drs. Stephen Jennings and Brian Wigdahl participated in the intellectual development and critically evaluated all aspects of the chapter. Dr. Stephen Jennings is supported by developmental funds provided by the Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine

5.1. Overall summary

The studies presented in this dissertation examined the lip scarification model of HSV-1 infection. The lip scarification model closely resembles the natural inoculation site for the majority of new HSV-1 infections in humans. Chapter II described studies of the events that occur during acute primary infection using the lip scarification model. Chapter III described studies to explore the potential for the lip scarification model to be used to study latency and reactivation. Finally, in Chapter IV studies were performed to develop a new therapeutic tool for treating acute HSV-1 infections using gene editing technology. This chapter summarizes our observations, discusses translational implications, and addresses future studies which should be performed.

5.2. The course of primary infection using the lip scarification model

Infected mice develop clinical lesions

Using the lip scarification model of infection, we examined mice at various time points for apparent clinical lesions at the site of inoculation. The vehicle treated animals showed no pathology associated with the scarification procedure. Additionally in virus treated animals, no pathology could be observed at the earliest time points observed. However large erythematous lesions appeared on the lower lip beginning 5 days post-infection. These lesions appeared to be vesicular and a crust could be

observed. Virus treated animals presented healing lesions on the lower lip 8 days post-infection. No clinical lesions could be identified on mice 15 days post-infection.

We processed the lower lips and trigeminal ganglia (TG) from mice sacrificed at selected time points after infection. Staining of tissue sections with Hematoxylin and Eosin (H&E) revealed a degraded and compromised epidermal layer that was then later covered with a proteinaceous crust and eventually restored and healed by 15 days post-infection. The degraded tissue contained viral inclusion bodies consistent with HSV-1 infections in humans. The healed dermal layer contained an abundance of retained leukocytes.

Immunohistochemistry revealed that HSV-1 antigen is initially found on the surface of the lip before being found abundantly in hair follicles of the dermal layer. HSV-1 antigen is not detectable in the lower lip 8 days after infection. $CD45^+$ leukocytes rapidly infiltrate the lip and associate with areas of tissue containing HSV-1 antigen. The $CD45^+$ leukocyte infiltration begins to contract by 8 days post-infection, but a large number are still present 15 days post-infection.

We showed by plaque assay that the peak of cell-free virus could be found in the lip between 2 and 5 days post-infection. No cell-free virus could be detected in the lip 8 days after infection. The peak of cell-free virus in the TG occurred at 5 days post-infection. This indicated a time

delay needed for the virus to travel from the site of inoculation to the TG. No cell-free virus could be detected in the TG 8 days after infection.

A genome copy assay was performed to determine the levels of HSV-1 genomes present in lip and TG at selected times. Consistent with the plaque assays, the highest viral genome copy number occurred in the lip at 5 days post-infection. Peak levels of genome copies in the TG were obtained 5 days post-infection. However, a steady state level of genomes was established in the TG which persisted through 15 days post-infection

These data demonstrate the ability of the lip scarification technique to model infection of the orofacial epithelial tissue. We were able to detect clinical lesions which were similar to lesions which appear on humans who are suffering reactivations. Similar to what is observed in patients, clinical lesions on mouse lips correlated with histopathological evidence of infection. Specifically, we observed well characterized HSV-1 viral inclusion bodies and a large proteinaceous crust covering areas of epidermal degradation. Treatment of lip sections with antibodies specific for HSV-1 antigen revealed that the virus accumulates to high levels in the hair follicles. This is an interesting finding as it demonstrates that the lip model can also be used to study the disease herpes folliculitis (Böer et al., 2006).

The time frame of acute infection by exhibited clinical lesions were consistent with that of human reactivations There are no prospective studies of human HSV-1 infections with associated histopathology, so the

process of lesion formation and resolution is unknown. A clinical study comparing achieved samples of human histopathology from HSV-1 infections to those of the mouse lip scarification model will confirm the accuracy of disease modeled. The model can then be used to study the formation and resolution of HSV-1 lesions in the orofacial epithelia.

After utilizing the model to characterize the process of lesion formation and resolution, the model can then be used to address more interesting questions. Current and future therapeutics can be analyzed by their effect on the histopathology of HSV-1 infections in the lip. New therapeutics will be measured not only in the time course of clinical lesions but also by reduction of tissue damage of the lip.

Another interesting question which has not been addressed by human clinical studies or other animal models is the nature of lesions caused by reactivations from latency. The data presented in this dissertation can inform us about the process of lesion formation from virus which is topically administered. The data in chapter 3 confirmed that a latent HSV-1 infection is established and is capable of reactivating. Inducing reactivation in vivo can allow us to determine the process of lesion formation and resolution from the endogenous latent HSV-1 infection. We can evaluate if changing the originating source of virus input affects the location and severity of pathology present in the tissue.

Leukocytes infiltrate the trigeminal ganglia

TG sections from mice sacrificed at selected times were stained with H&E. We did not observe any specific pathology in neurons from infected animals. Cellular infiltration was observed beginning 5 days post-infection. We saw that infiltrating cells associated with and surrounded neuronal cell bodies at 8 and 15 days post-infection. Immunohistochemistry confirmed the infiltrating cells were $CD45^+$ leukocytes responding to HSV-1 infection of the TG. The $CD45^+$ cells surrounded neurons in the TG and were retained through 15 days post-infection. We also determined that $CD3^+$, $CD4^+$, and $CD8^+$ T cells can be found in the TG 15 days post-infection.

These data are important for demonstrating the immune response to HSV-1 infection in the lip involves TG infiltration with leukocytes. There is a large body of evidence which demonstrates that $CD4^+$ and $CD8^+$-T cells can be found in the TG (Khanna et al., 2003; Liu et al., 1996). Mouse in vivo studies further confirmed that $CD8^+$ T-cells play a role in maintaining the virus in a latent state (St Leger and Hendricks, 2011). Disruption of $CD8^+$ T-cell function or the pool of $CD8^+$ T-cells accelerated reactivation from latency (Cherpes et al., 2008; Freeman et al., 2008). This can be corroborated in humans because stress inhibits $CD8^+$ T-cell function and people suffering high levels of stress consistently have higher rates of reactivation (Glaser and Glaser, 1998).

Further studies will allow us to define the immune cells which comprise this acute phase TG infiltration. We can establish a time course of infiltration from different populations of immune cells. We can further establish the contraction of immune response and determine what specific cell populations are likely to

remain retained in the TG. These studies will be useful for studies to determine the nature of Tissue Resident Memory T-cells (T_{RM}).

5.3. Latency and reactivation from mice inoculated via lip scarification

We processed lip and TG tissue from mice sacrificed 30 days post-infection and stained with H&E. There were no signs of pathology present in the lip 30 days post-infection. The epidermal layer was intact and not enlarged. Immune cells could be found retained in the lip 30 days after infection, and immunohistochemistry revealed them to be $CD3^+$, $CD4^+$, and $CD8^+$ T-cells. Cells bearing these markers could be found in hair follicles where a large accumulation of HSV-1 antigen was observed during acute infection. Immunohistochemistry performed on TG sections revealed that $CD3^+$, $CD4^+$, and $CD8^+$ T-cells could be found in the TG. The T cells were found closely associated with neurons.

This is an important result that correlates the immune response in the lip model to responses seen in other mouse models and human post-mortem samples (Held et al., 2012; Khanna et al., 2003; van Velzen et al., 2013). The role of T-cells in maintaining the virus in the latent state has not been firmly established. Some investigators have suggested that $CD8^+$ T-cells in the TG are not involved in maintaining the virus in the latent state (Held et al., 2011). Other investigators have demonstrated that inhibition of T cells from latently infected tissue leads to accelerated reactivation from latency (Liu et al., 2000).

The presence of $CD4^+$ and $CD8^+$ T-cells in the lip and TG suggests that the lip scarification model can be used to study the formation and role of immune cell populations maintained in tissue. We hypothesize that the $CD8^+$ T-cells in the lip and TG belong to the T_{RM} class of memory T-cells. In contrast to $CD8^+$ effector memory T cells, these cells will remain retained in the tissue where antigen was first encountered. The proximity to these cells ensures that memory immune cells will be available to rapidly respond to reexposure to antigen in tissue.

T_{RM} cells have been identified in numerous tissues including the brain (Casey et al., 2012), intestines (Masopust et al., 2006), lungs , liver (Tse et al., 2013) and recently in the spleen and lymph nodes (Schenkel et al., 2014). T_{RM} cells arising from HSV infections have been confirmed in the skin (Mackay et al., 2013), dorsal root ganglia (Gebhardt et al., 2009) and female genital tract (Iijima and Iwasaki, 2014). A distinctive tissue pattern was seen in T_{RM} cells of the skin in mice following HSV-1 challenge. $CD4^+$ T_{RM} cells were found localized in the dermis while $CD8^+$ T_{RM} cells were found in the epidermal layer (Mackay et al., 2013). Both $CD4^+$ and $CD8^+$ T_{RM} cells could be found in hair follicles. The results of our immunohistochemistry experiments in the lower lips of mice following HSV-1 inoculation closely match this tissue localization. The $CD8^+$ T-cells in the lip were found in either the epidermis of hair follicles. The $CD4^+$ T-cells in the lip were found in the dermal layer or in the hair follicle.

The canonical markers used for identifying T_{RM} cells are CD69 and CD103. CD69 is commonly known to be a recent activation marker in T cells, but also serves to downregulate expression of genes which help cells migrate out of tissues (Skon et al., 2013). CD103 is an integrin receptor which binds to e-cadherin. It is hypothesized that this interaction helps cells to remain in the tissue where antigen was first encountered. Flow cytometry is the best method for determining the variety of markers present on cells in tissue. To confirm T_{RM} cells are retained in the lip and TG, we would need to demonstrate CD69 and CD103 expression. T_{RM} cells have decreased expression of the chemokine receptor CCR7 and sphingosine 1 phosphate receptor (S1PR1) which receives signals that induce tissue exit and circulation. Additionally cell tracing experiments should be done to confirm that these cells are not being seeded from the circulation. T_{RM} cells are believed to be homeostatically quiescent meaning that they are not actively replicating to replenish their population, instead consisting of a long lived population. To confirm this we would analyze cells for the presence of the replication marker Ki-67 in cells isolated from the lip and TG.

The timing of T_{RM} cell generation is an interesting question which can be addressed using this model. One theory is that T_{RM} cells are generated from differentiation of memory T cells which are already present in the peripheral tissue site where antigen is encountered. Preliminary data from our lab demonstrate $CD69^+$ $CD103^+$ $CD8^+$ T-cells in the TG 5 days post-infection (unpublished data). These results could point to an alternative hypothesis that

T_{RM} cells are seeded in the peripheral sites during the acute phase and are retained following contraction of the immune response. T_{RM} cells have been found in the spleen and lymph node of mice. These cells could be a source of T_{RM} cells seeding into the peripheral sites. The lip scarification model can be used to explore these alternative hypotheses.

The TGs from latently infected mice harbor HSV-1 genomes

We isolated DNA and RNA from the lips and TGs of mice sacrificed 30 days post-infection. We did not detect any HSV-1 genomes or LAT expression in the lips from latently infected mice. This was not surprising in the context of our current understanding of peripheral HSV-1 infections. However, it did not completely eliminate the possibility that latent HSV-1 genomes existed in the lip from mice. It is possible that the number of latent genomes present within the lip was below the level of detection using these techniques.

Trigeminal ganglia from these mice had no infectious cell-free virus, but had HSV-1 genomes present in levels similar to what was observed 15 days post-infection. We were also able to detect LAT expression in these tissues, providing future evidence that latency has been established in these mice 30 days after infection.

Cultured TGs from infected mice reactivate from latency

We cocultured TGs from mice sacrificed 30 days post-infection with Vero indicator cells. During the 10 day culture period, the Vero cell monolayer began displaying cytopathic effect (CPE) consistent with *in vitro* HSV-1 infections. H&E

staining of the Vero cell monolayer demonstrated viral inclusion bodies consistent with HSV-1 infection. Media sampled 4-6 days after the initial coculture plating produced plaques on new Vero monolayers which indicated reactivation had occurred in the TGs. The plaques were positive for HSV-1 antigen and had morphology consistent with stock HSV-1 McKrae. These results provide evidence that TGs from infected mice harbor latent HSV-1 that is reactivating. The plaque morphology indicates that the virus produced from these cultures is the same as the strain of HSV-1 used to inoculate mice.

Cultured lips from infected mice reactivate from latency

We cocultured lip tissue from mice sacrificed 30 days post-infection with Vero indicator cells. As with the TG coculture, during the 10 day culture period, the Vero cell monolayer began displaying CPE consistent with HSV-1 infections *in vitro*. H&E staining of the Vero cell monolayer demonstrated viral inclusion bodies consistent with HSV-1 infections. Media samples from 5-7 days after plating produced plaques, indicating reactivation occurred. The plaques were positive for HSV-1 antigen and had morphology consistent with stock HSV-1 McKrae. These results provide evidence that lips from infected mice harbor latent HSV-1 that is reactivating. The plaque morphology indicated that the virus produced from these cultures was the same as the strain of HSV-1 used to inoculate mice.

There are still many more questions which need to be addressed to thoroughly characterize latency and reactivation in the lip scarification model of infection. We used only a single dose infection and demonstrated that we could

detect latent HSV-1 genomes in the TG, LAT expression, and a capability for latent genomes to reactivate. Additionally we were able to detect reactivation in lips that were cocultured with Vero cells after sacrifice. However we were not able to detect latent HSV-1 genomes or LAT expression in nucleic acids isolated from the lips of mice infected. It is possible that any latent HSV-1 genomes present in the lip are below a level that we can detect. Future experiments exploring higher doses of input virus can help us determine an optimal concentration for infection. Higher input dose of virus is correlated with an increased level of latent HSV-1 genomes in the TG. An ideal dose would allow mice to recover from acute infection without any CNS disease, no persistent replication in the tissue, and an increased number of HSV-1 genomes in the lip and TG. If we are still not able to detect latent HSV-1 genomes in the lip, we can use a nested PCR approach which has a higher sensitivity for low levels of target DNA.

A larger level of latent HSV-1 genomes are also correlated with an increased frequency of reactivation. This will allow us to conduct more quantitative experiments into the nature of latency and reactivation in the lip and TG. Infectious center assays can be used to measure the number of infected cells in the lip and TG which harbor latent HSV-1 genomes. We can localize the latent HSV-1 genomes in the lip and TG tissue sections using in situ hybridization. This is an important experiment which can reveal what cells are harboring latent HSV-1 genomes in the lip. Culture experiments on recently

dissected lips and subsequent histological processing can also reveal the process of reactivation in the lip.

With these data demonstrating retention of T cells in tissue and the ability of explanted tissue to reactivate, we can begin to explore the role that immune cells play in maintaining the virus in the latent state. An important first experiment will be to demonstrate that T cells associate with cells harboring latent HSV-1 genomes in the lip and TG. Explanted and digested tissue can be treated with antibodies that will inhibit the activity of $CD4^+$ and $CD8^+$ T-cells in culture. The rate and intensity of reactivation will inform reveal the effect these cells are having on maintaining latency. In vivo experiments which deplete specific T cell populations can also be used to explore the effect of immune cells on maintenance of latency.

Finally we will explore in vivo reactivation studies in mice inoculated through the lip scarification model of infection. Being able to induce in vivo reactivation is important for the model to be appropriate for an animal model to study latency and reactivation. Common triggers of in vivo reactivation in mouse models include restraint stress, hormone injections, and variable temperature stressors. In addition to these triggers, we will explore triggers of reactivation that act on a local level. A second treatment of lip scarification might be enough to trigger a reactivation event in the lip. Additional local triggers can be ultraviolet (UV) light inactivated viruses that can induce a memory immune response and capsaicin.

5.4. Delivery of CRISPR/Cas9 gRNAs targeting HSV-1 immediate early genes using AAV vectors.

CRISPR/Cas9 gene editing has the potential to lead to a curative therapy for latent HSV-1 infections. We tested delivery of CRISPR/Cas9 gRNAs via adeno-associated virus (AAV) vectors. We injected a cocktail of AAV vectors which encode gRNAs targeting the HSV-1 immediate early genes ICP0 and ICP27. We measured the expression of the gRNAs in the lip and TG over the course of 3 days. We determined that both the ICP0 and ICP27 gRNAs had the highest level of expression 24 hours after injection in the lip and TG. We tested two different doses of AAV injections and found differential expression patterns in the lip and TG. The high dose AAV injection had the highest expression in the lip and the low dose AAV injection had the highest expression in the TG. Expression of gRNAs from both gRNAs at both doses declined at time points after 24 hours. It is possible that the difference of expression of gRNAs from the different doses is related to negative effects of injecting such high amounts of AAV particles. Additionally it will be important to determine what cells specifically are expressing the gRNAs in tissue. In order for the CRISPR/Cas9 endonuclease to be able to act on latent HSV-1 genomes, it must be delivered to neurons which are harboring the latent virus. Combining injection of an AAV expressing eGFP with immunohistochemistry, we can detect GFP expression in a specific cell types in tissue. Another consideration that must be addressed is multiplexing different gRNA targets into a single AAV particle to ensure that a single transduced cell is expressing multiple gRNAs.

5.5. Implications for research in HSV-1 animal models

The studies presented in this dissertation provide much needed information about the lip scarification model of HSV-1 infection. Inoculation through the lip models is a physiologically relevant site for new HSV-1 infections in humans. We sought to establish the parameters of acute and latent infection in order to use this model for studying HSV-1 disease in the orofacial epithelial tissue. We found that inoculation through the lip closely resembles clinical and histopathological observations previously observed in human infections. We demonstrated that the kinetics of virus replication in the lip and TG were (Hoshino et al., 2007) similar to those observed in other animal models (Fig. 5.1) These results suggested that the lip scarification model was an appropriate system for studying acute HSV-1 infections and models human infections in a relevant tissue site.

Figure 5.1. Model of acute infection following lip inoculation. Following scarification of the lower lip, stock HSV-1 was administered to the scratched site. The virus was adsorbed and initiates productive infection in the periphery. During replication in the lip, the virus infected innervating sensory neurons and gained access to the trigeminal ganglia. During the initial infection, viral antigen was delivered to the sub-mandibular draining lymph node and the anti-viral immune response was initiated. $CD45^+$ leukocytes infiltrate the lower lip and trigeminal ganglia. Following immune cell infiltration, infection in the lip and trigeminal ganglia resolved. Infected neurons harbor HSV-1 genomes after the resolution of replicating virus. $CD45^+$ cells surround neurons and are retained after resolution of replicating virus.

Figure 5.1.

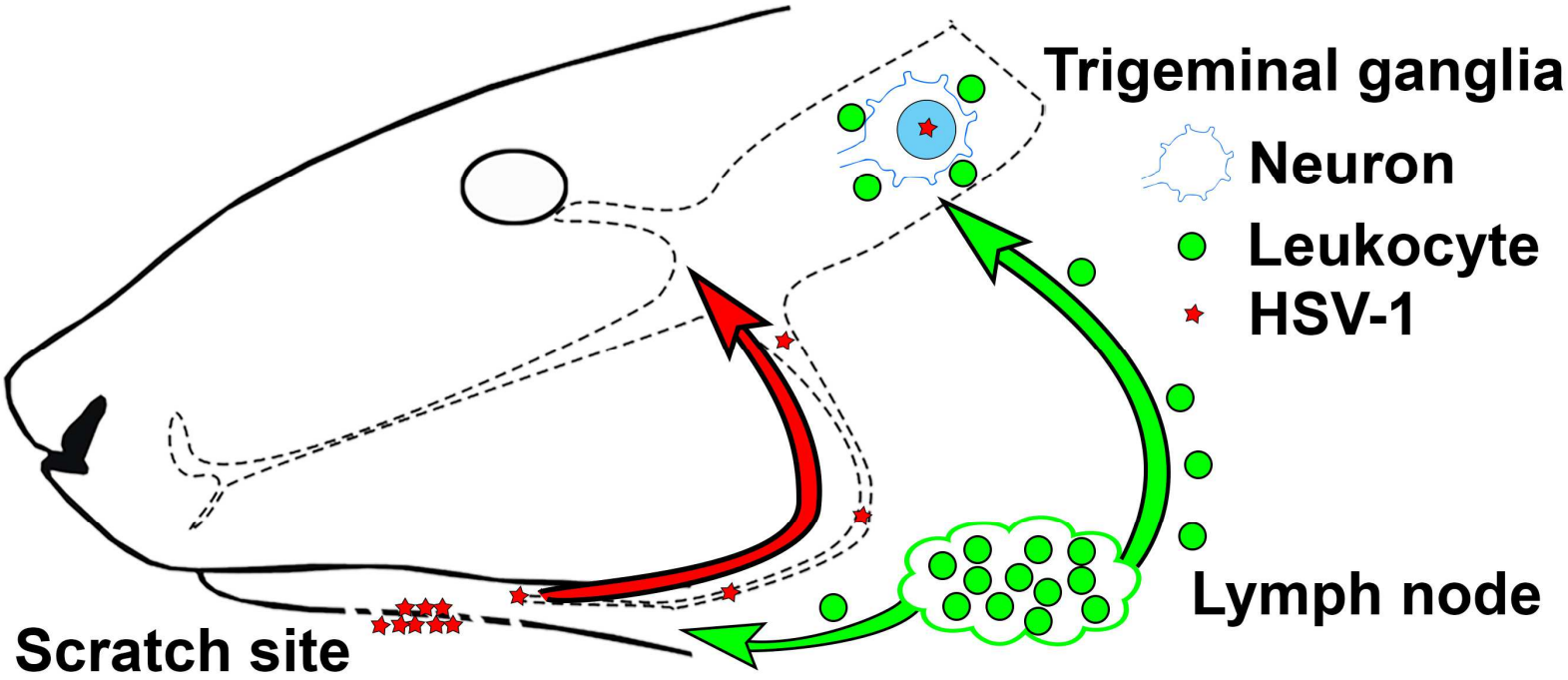
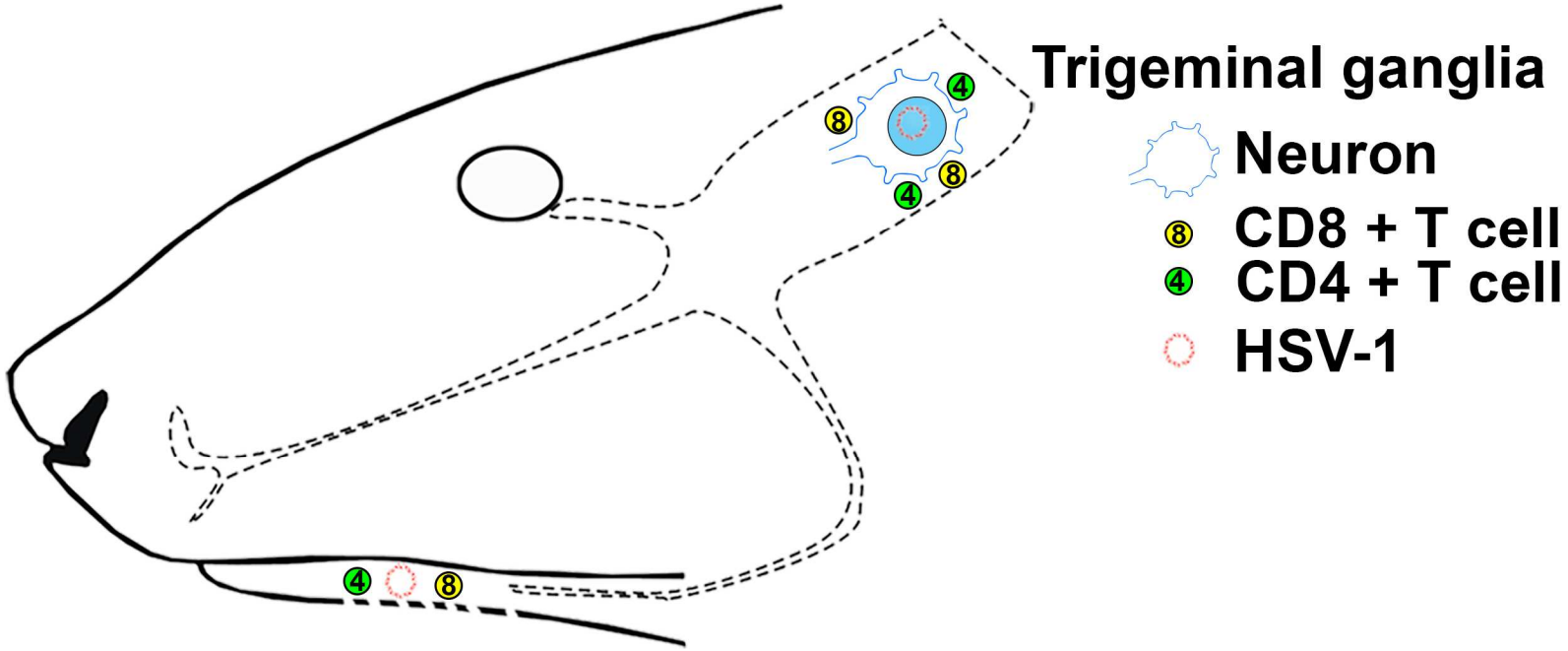

Trigeminal ganglia
Neuron
Leukocyte
HSV-1
Lymph node
Scratch site

Figure 5.2. Model of latent infection following lip inoculation. After resolution of primary infection, the virus establishes a latent infection in the trigeminal ganglia (TG). HSV-1 DNA and LAT transcripts can be recovered from the TG of infected mice. Tissue from both the lip and TG was capable of reactivating from latency when cultured after sacrifice. Neurons in the TG are surrounded by CD4+and CD8+ T-cells. In the lip, CD4+ and CD8+ T-cells can be located in the dermis and hair follicles. These T-cells are likely responding to low level viral antigen being produced from latently infected cells.

Figure 5.2.



We further determined if the lip scarification model could reproduce latency and reactivation (Fig 5.2.). We found that at time periods in which the virus should be considered latent based on studies performed by other investigators, there indeed was no replicating virus present in the lip or TG. The TG harbored latent HSV-1 genomes and when cultured were capable of reactivating from latency. The virus recovered from cultured samples was positive for HSV-1 antigen and produced plaques morphologically similar to the input stock HSV-1 McKrae strain.

Surprisingly, we found that virus could be recovered when the lips from infected animals were similarly co-cultured. This novel observation suggests that lip scarification could be an appropriate model for studying peripheral latency. Peripheral latency has been suggested from clinical observations in corneal transplant patients (Higaki et al., 2015; Kaye et al., 1991). It has been reproduced with varying levels of success in mouse and rabbit models (Cook and Brown, 1986; Higaki et al., 2015) . There are interesting clinical implications to peripheral latency in the lip. Is it possible that cases of human reactivation can be initiated in both the TG and in the lip? It could also be that infectious reactivated virus is being retained in the nerve endings from neurons in the TG, awaiting only an appropriate trigger to be released into the peripheral environment. Further experiments are needed to confirm the presence of latent HSV-1 genomes being harbored in the lip. Additionally, the molecular basis of latent genomes in the lip needs further exploration.

Peripheral latency could exist in a form different from what is seen in the TG, thus eluding detection.

Finally we explored delivery of CRISPR/Cas9 gRNAs into relevant tissues by AAV vectors. We found that after local delivery of AAV vectors into the lower lip, we could detect gRNA expression in both the lip and TG. The highest level of expression of the gRNAs was 24 hours after injection. These results confirm that timing of AAV vector injection is going to be critical for studying CRISPR/Cas9 gene editing of the HSV-1 genome. We can also exploit this timing to determine the effect of CRISPR/Cas9 in different tissue sites.

It would be quite interesting if CRISPR/Cas9 were capable of reducing virus replication during acute infection. A reduction in viral load in the lip could mean a reduced viral load in the TG. A reduced viral load entering the TG would reduce the number of infected neurons and reduce the capability and frequency of reactivation (Hoshino et al., 2007). However, CRISPR/Cas9 treatment is not likely to be implemented during primary acute infection. Most people are unaware of when they are first exposed until symptoms of primary infection occur. By that time, the virus has already infected the innervating sensory neurons and gained access to the TG.

The most promising course of treatment for HSV-1 infections using CRISPR/Cas9 would be to target the latent HSV-1 genome maintained in neurons. If the CRISPR/Cas9 and gRNAs could be effectively delivered and expressed in the neurons, the latent HSV-1 genome could be edited.

Recurrent infections could be eliminated by targeting HSV-1 genes necessary for reactivation. It is also conceivable that the genome could be targeted in a way to cause large portions of the genome to be deleted. Similar approaches are being used to target integrated human immunodeficiency virus type 1 (HIV-1) in infected T cells. The integrated virus has identical copies of the long terminal repeat (LTR) flanking the viral genome in T cells (Kaminski et al., 2016). By targeting the LTR sequence on both sides of the viral genome, double-stranded break repair mechanisms will remove the integrated viral genome. The latent HSV-1 genome must be examined for similar sites with appropriate protospacer sequences that will make those sites accessible to Cas9 mediated cutting.

The value of examining CRISPR/Cas9 treatment during acute infections lies in optimizing the design and delivery of Cas9 and gRNAs. To only look for efficacy in CRISPR/Cas9 treatment in mice which already have established a latent infection would be prohibitive in both time and money. The design of gRNAs and the vehicle used for delivery can be evaluated *in vivo* during the acute infection. We can analyze infected tissue samples for evidence of genome editing occurring in the HSV-1 genes targeted by the gRNAs. This information would confirm that the Cas9 and gRNAs are delivered and expressed at usable levels.

The *in vivo* acute infection information will inform the eventual experiments focusing on editing the latent HSV-1 genome. Mice which are latently infected can be injected with the CRISPR/Cas9 treatment through either the lip or

systemically through the intraperitoneal or intravenous routes. The genomic editing of the latent HSV-1 genome can be assessed with the surveyor assay from infected tissue. The most important experiment will be to compare reactivation frequencies from mice which received CRISPR treatment compared to those receiving empty vehicle. A significant reduction in reactivation from treated mice would suggest that CRISPR therapy holds promise for treating latent HSV-1 infections in humans.

In conclusion, we characterized the acute events of HSV-1 infection following inoculation of the lower lip in mice. We found that mice were productively infected and had pathology consistent with human infections. The virus gained access to the TG and established a latent infection. The virus was capable of reactivating from latency to produce new infectious virions in culture. The development and characterization of a biologically relevant mouse model of HSV-1 infection in the lip is a crucial first step to the development of novel therapeutics and interventions, such as the CRISPR-mediated removal of HSV-1 genomes attempted in Chapter IV.

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